THE NATURE AND ORIGIN OF WHEAT STEM GLUCOFRUCTANS

by

Leighton Eugene Lopatecki

DEPARTMENT OF PLANT SCIENCE

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THE NATURE AND ORIGIN OF WHEAT STEM GLUCOFRUCTANS

A DISSERTATION

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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by

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ABSTRACT

Studies were made of the nature, origin, and accumulation of glucofructans in wheat stems. The following conclusions were drawn:

1. Glucofructans of wheat stems are non-reducing fructose polymers containing one molecule of glucose. Fructose units are almost certainly present in furanose form and joined by beta linkage. The molecular size ranged from three to about 10 hexose units.

2. Most of the glucofructans are extracted with alcohol; the remainder with water.

3. Chromatographic evidence suggests that the alcohol soluble compounds form two homologous series, both series being fructose polymers of sucrose. The first members of the series were tentatively identified as the trisaccharides F^6-B-fructosylsucrose and F^1-B-fructosylsucrose.

4. One major component is present in the water soluble glucofructan. Features of acid and invertase hydrolysis indicate that the compound is in the form of a chain rather than a ring, and possibly contains a sucrose unit.

5. Only one glucofructan, probably identical to F^1-B-fructosylsucrose, is synthesized *in vitro* by invertase digests of wheat stems or leaves.

6. An unknown substance present in maturing wheat

stems inhibits hydrolysis of sucrose by invertase without affecting transfructosidase activity. This compound probably facilitates accumulation of glucofructans in wheat stems, but is not essential for glucofructan synthesis.

7. Higher polymer glucofructans are synthesized by an unknown enzyme mechanism. No evidence was obtained that glucose-6 phosphate, fructose-1 phosphate, fructose-1 -6 diphosphate or adenosine triphosphate were involved in this synthesis.

8. Accumulation of glucofructan in wheat stems commences around the stage of heading, reaches a peak at the milk stage, and then declines rapidly as the plant matures. At peak concentration, glucofructans comprised 9.5 to 15 per cent of the dry weight of stems, and 50 to 70 per cent of total sugar.

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THE NATURE AND ORIGIN OF WHEAT STEM GLUCOFRUCTANS

INTRODUCTION

Carbohydrates of the wheat plant have been studied in relation to a number of distinct projects. Blish, Sandstedt and Astelford (27) and Koch, Geddes and Smith (66) examined the sugars of wheat grain and flour in relation to the fermentation of dough. A possible relationship between carbohydrates and winter hardiness stimulated research on sugars of the wheat plant by Newton (86) and by Janssen (63). Again, Roberts (102) investigated the sugars of young wheat leaves in an attempt to determine the metabolic basis for the respiratory gradient characteristic of wheat leaves. More recently, Lopatecki, Longair and Farstad (75), surveyed the soluble carbohydrates of wheat stems to elucidate, if possible, the mechanism of resistance against the wheat stem sawfly, *Cephus cinctus*, Nort.

From the latter study (75), it appeared that toward maturity, oligosaccharides accumulated in wheat stems to a rather remarkable degree. Preliminary study of these oligosaccharides showed that the group was hydrolysed rapidly by dilute acid or invertase, and produced only fructose and glucose.

From the above evidence, two tentative conclusions

were drawn. First, it seemed probable that the oligosaccharides of wheat stems were related to the glucofructosans formed from sucrose by invertase of yeasts, moulds, and some higher plants (11, 13, 22). Secondly, it appeared that special conditions must exist within the wheat stem to foster accumulation of these compounds.

The purpose of the present work is to present evidence supporting both the above conclusions.

LITERATURE REVIEW

Terminology

Since carbohydrate terminology is not yet completely standardized, some of the terms used in the present work are defined here.

Following Pigman and Geopp (94), the term "oligosaccharide" includes the disaccharides and other polymers of simple sugars containing 2 to 10 monosaccharide residues. The term "sugar" includes mono- and oligosaccharides, but not polysaccharides.

Following Whistler and Smart (113), fructose polymers of microorganisms are called "levans", while similar compounds from higher plants are termed "fructans". The term "fructosan" is used to designate this general class, in place of the more common polyfructosan, since the latter

connotes polymerized fructosan.

Similarily, polymers of glucose are termed glucans, while "dextran" is reserved to apply specifically to glucans formed by bacteria from sucrose (113). "Glucosan" is used as a general term for both classes.

No common, yet specific, term exists for the monoglucopolyfructosan oligosaccharides of microorganisms and of higher plants. Since these oligosaccharides contain one unit of glucose in the molecule, they are, strictly speaking, not fructans. The term "monoglucopolyfructosan" is, however, cumbersome, hence various alternative terms have been used. MacLeod (76) suggested "low molecular fructosan", or simply "polyfructosan". Others have used the general term "oligosaccharides" (2, 14, 41). In the present work the more precise term of Bhatia, et al (23) "glucofructosan" is preferred. When reference is made specifically to compounds of higher plants, the term "glucofructan" of Boggs and Smith (29) is used, since this agrees with the nomenclature already adopted.

Simple Sugars and Glucofructans from Wheat Leaves and Stems

Although considerable information is available on the sugars of wheat leaves, particularly from young plants, relatively few studies have been made on the sugars of wheat stems. Roberts (102) and Ward (108) have shown that at the first leaf stage, sucrose is the main carbohydrate

of leaves of Kapli wheat. Bidwell, Krotkov and Reed (24) found that wheat leaves picked in June possessed free sugar, mainly as sucrose, with traces of glucose and fructose. Bradfield and Flood (30) found that leaf blades and sheaths from maturing Square Head wheat contained fructose, glucose, sucrose and sorbitol. Additional unresolved chromatographic spots indicated five or more carbohydrates of higher molecular weights. The distribution of carbohydrates between leaves and stems was not determined. More recently, Lopatecki, Longair and Forstad (75) presented evidence that glucose, fructose, and sucrose appear in wheat stems and leaves from early shot blade until maturity. Following shot blade, a series of non-reducing oligosaccharides appeared in both stems and leaves. Complete hydrolysis of these oligosaccharides by invertase produced glucose and fructose only, while chromatographic mobility suggested a molecular size of three to ten hexose units.

Glucofructans from Other Sources

Edelman (40), and Blanchard and Albon (26), found that glucofructan oligosaccharides are formed from sucrose by extracts of yeasts and moulds. The following year, Kohanyi and Dedonder (67) reported that a levan-forming enzyme of Bacillus sublilis produced a series of non-reducing oligosaccharides from sucrose.

Subsequently, glucofructan oligosaccharides have been

identified from a variety of higher plants. Bacon and Edelman (15) showed that artichoke contains in addition to inulin, a series of such compounds. A group of glucofructans has been found in wheat endosperm (66, 116), in barley grain (76), and in apricot fruit (6). The presence of two homologous series of oligosaccharides have been reported by White and Secor (114) in wheat flour. The stems of Agave vera cruz, a rich source of polyfructosans, also possess lower molecular weight oligosaccharides (22). A glucofructan has been isolated recently from the Hawaiian ti plant (29).

Bidwell, et al (24), made a chromatographic examination of leaf extracts from 27 families of Spermatophyta. They reported the presence of alcohol soluble fructosides in the following six families: Gramineae, Compositae, Oleaceae, Polygonaceae, Primulaceae and Scrophulariaceae. The stage of growth was not recorded when the samples were taken. However, the trace of fructoside reported in leaves of Triticum vulgare suggest that this was a very young sample. It has been shown recently that at stages approaching maturity, approximately one half the total sugars of wheat leaves is in the form of glucofructans (75). Consequently, samples taken from nearly mature plants might present a much more general distribution of glucofructans.

Biosynthesis of Glucofructans

Information on the biosynthesis of glucofructans has come almost exclusively from studies on microorganisms. Edelman (40), and Blanchard and Albon (26), first reported in 1950 that oligosaccharides are formed from sucrose by enzymes of yeasts and moulds. This work was subsequently confirmed by Bacon (9, 10), and Bealing (19, 20, 21). Since other enzyme activity, that of sucrose hydrolysis paralleled glucofructan synthesis, seemingly only one enzyme was involved. This single enzyme was considered to be invertase.

That a second independent activity of invertase should pass notice for so many years deserves comment. This feature was missed undoubtedly because of the transitory appearance of glucofructans under *in vitro* conditions. The compounds formed through invertase activity are ultimately hydrolysed to glucose and fructose by the same enzyme. Not until the advent of paper chromatography was anything unusual apparent in the hydrolytic procedure. Subsequent work suggests that perhaps in no case is the activity of invertase limited simply to the hydrolysis of sucrose.

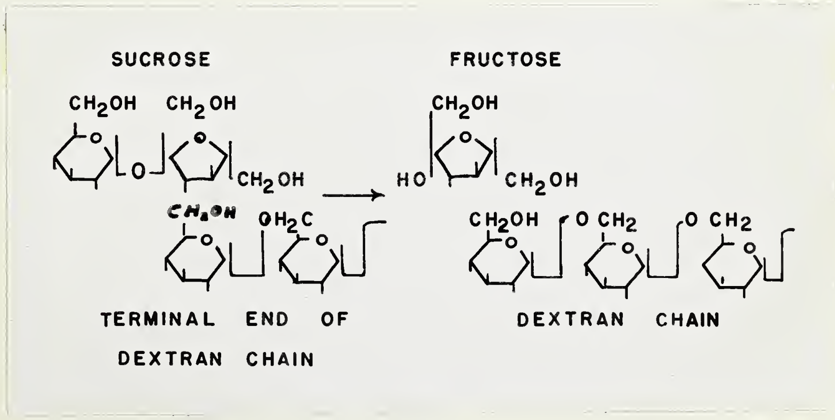
Indication of the mechanism of glucofructan synthesis first followed Blanchard and Albon's (26) examination of sugar content of a trisaccharide synthesized by invertase. The presence of two fructose to one glucose unit suggested

to Fisher, et al (45) a transfructoside synthesis, according to the following scheme:

1. sucrose + enzyme ⇌ fructose - enzyme + glucose
2. fructose - enzyme + sucrose ⇌ trisaccharide + enzyme
3. fructose - enzyme + water ⇌ fructose + enzyme.

Both sucrose and water compete as acceptor for the fructose unit being transferred. Thus, at high sucrose concentration, synthesis of trisaccharide is favoured; at high water concentration, hydrolysis is favoured. This requirement for high sucrose concentration for synthesis is undoubtedly another reason why the synthetic activity of invertase was overlooked for so long.

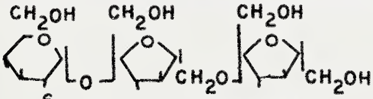
The first two stages of the above scheme are analogous to the transglucoside synthesis of dextran from sucrose first described by Hehre in 1941 (55). This synthesis, mediated by enzyme extracts of Leuconostoc mesenteroides, may be illustrated as follows:



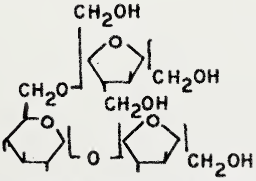
In a similar fashion, extracts from *Streptococcus salivarius* (56) and *Bacillus subtilis* (57) produce levans from sucrose. This transglycosidase synthesis, termed polymerative sucrolysis by Hestrin (57), involves neither water nor phosphate directly. The direction of reaction toward synthesis is favoured thermodynamically (39).

Subsequent work has added detail to the transfructosidase mechanism of yeast and mould invertase. Bacon (9, 10), and Wheland and Jones (110), showed that sucrose could be substituted as acceptor by a variety of alcohols, free sugars and sugar alcohols. The invariable requirement was that of a primary alcoholic group.

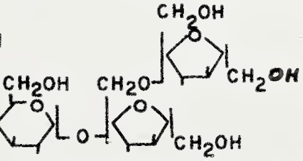
This finding suggested that three trisaccharides might be formed from sucrose, following substitution of each of the three primary alcoholic groups in the sucrose molecule. Bacon (11), and Gross, Blanchard and Bell (52), found that yeast invertase produced three such compounds. These are illustrated below.



1. F^6-B-FRUCTOSYLSUCROSE



2. G^6-B-FRUCTOSYLSUCROSE



3. F^1-B- FRUCTOSYLSUCROSE

Furthermore production of one or more of these substituted sucroses was found to be a feature of invertase from plant sources. Thus, Bacon and Bell (13) had previously identified F^1-B-fructosylsucrose as a product of mould invertase. Later, Allen and Bacon (2) found F^1 and G^6-B-fructosylsucrose to be produced by invertase from sugar beet leaves. However, Feingold, et al (44) showed that formation of fructosylsucroses was not entirely limited to invertase activity. These workers identified F^1 and F^6-B-fructosylsucrose as products of levan sucrase.

Variation in fructosylsucroses synthesized by different invertases pointed to a variation in the enzyme molecule. A further variation became apparent when glucose, rather than sucrose, was used as acceptor. Here mould invertase formed sucrose while yeast invertase produced a reducing glucofructoside (11).

Hestrin, et al (58) were able to resolve to some extent differences in activity of invertase from yeasts and moulds, and levan sucrase from *Aerobacter levanicum.* This was achieved by postulating a difference in bond energy of the various enzyme-substrate complexes. Recent work by Burton and Krebs (36), and by Hehre (56), suggested that the free energy change associated with hydrolysis of sucrose was double that of a polyglucose or polyfructose bond. Thus, the formation of a trisaccharide by fructose transfer from

sucrose is an irreversible downhill process.

Hestrin, et al (58) postulated that the high bond energy of sucrose was conserved in the enzyme-fructose complex of mould invertase and levansucrase, but not with yeast invertase.

Using Lipmann's notation for a high energy bond, the differences suggested by Hestrin, et al (58) and by Edelman (41) are reviewed below:

for yeast invertase:

$$\mathrm{F{\sim}G + enz \rightarrow F - enz + G}$$

$$\mathrm{F - enz + F{\sim}G \rightleftharpoons F - F{\sim}G + enz}$$

$$\mathrm{F - enz + H_2O \rightarrow F - enzyme}$$

$$\mathrm{F - enz + G \rightleftharpoons F - G -}$$

for mould invertase and levansucrase:

$$\mathrm{F{\sim}G + enz \rightleftharpoons F{\sim}enz + G}$$

$$\mathrm{F{\sim}enz + F{\sim}G \rightarrow F - F{\sim}G + enz}$$

$$\mathrm{F{\sim}enz + G \rightleftharpoons F{\sim}G}$$

additionally for mould invertase, but not levansucrase:

$$\mathrm{F - F{\sim}G + enz \rightleftharpoons F - enz + F{\sim}G}$$

$$\mathrm{F - enz + H_2O \rightarrow F + enz}$$

where F = fructose; G = glucose; enz = enzyme;

$\sim$ = high energy bond of Lipmann; $\mathrm{F{\sim}G}$ = sucrose;

$\mathrm{F - G -}$, = reducing disaccharide; $\mathrm{F - F{\sim}G}$ = fructosyl sucrose.

This scheme indicates that mould invertase resembles

levansucrase, in that fructose can be transferred reversibly from sucrose to enzyme. On the other hand, mould invertase resembles yeast invertase in its ability to hydrolyse fructosyl sucrose. Thus, in the former reaction, mould invertase forms a high energy substrate enzyme complex, and in the latter, a complex of low energy type. Hestrin, et al (58) suggest, therefore, that two enzymes may be present in mould invertase, a hydrolase, and a transfructosidase. A recent publication from Japan supports this view (80).

Glucofructans of larger molecular weights are also produced by invertase of yeast and moulds, and by levansucrase (41, 44, 58). Difficulties in purification and insufficient supply have so far precluded structural determinations by rigid classical methods. The available evidence suggests that these compounds are formed by further fructose transfer from sucrose to the growing glucofructan polymer (41, 44, 58).

It is significant that while levansucrase continues the polymerization process until a polysaccharide is developed, both yeast and mould invertases produce only oligosaccharides. Hestrin, et al (58) have suggested this difference may follow since levansucrase alone of the three enzymes is unable to hydrolyse the glucofructan chain which it forms.

Although not yet examined in so much detail, the

synthesis of fructosyl sucroses in higher plants appears analogous to that described with yeast invertase. Thus, Allen and Bacon (2) found that fructosyl sucrose synthesis from sucrose was catalysed by extracts from leaves, shoots and roots of a variety of plants. The synthetic activity was always related to the invertase activity of the enzyme preparation.

Incorporation of radioactivity from glucose into sucrose by invertase from sugar beet leaves was found too small to be of physiological significance (14). This suggests that sugar beet invertase, like yeast invertase, forms a low energy type of enzyme-substrate complex. Unlike yeast invertase, however, enzymes from higher plants do not form significant amounts of tetra or higher oligosaccharides (2). Seemingly, then, either in vivo conditions are more favourable for synthesis of larger oligosaccharides in higher plants, or some other mechanism is responsible for formation of these compounds.

On the basis of comparative biochemistry, a reasonable alternative synthesis for monoglucofructans is a phosphorylative mechanism analogous to that synthesizing starch from glucose-1 phosphate (54). However, a mechanism of this type has not yet been reported. It is significant perhaps that the sugar phosphate required in this synthesis, fructose-2 phosphate, has never been found in nature (94).

Methods of Oligosaccharide Analysis

Methods of extraction of oligosaccharides from plant tissues are similar to those for other sugars. Thus, enzymes of the plant material must be inactivated as rapidly as possible, and precautions taken during extraction and storage to prevent hydrolysis of sugar (71). Bathurst and Allison (17) have shown that fresh plant material may be inactivated with hot alcohol, and can then be stored conveniently in that medium without appreciable change in disaccharides. Methods of extraction, whether with hot or cold alcohol or with water are not critical (70).

The classical method of clarification of sugar extracts is a barium hydroxide or lead acetate treatment (34). More recently, ion exchange resins have been recommended for this purpose (115, 117, 118). Consideration must be given to the types of resins employed, however, since degradation of sugars can occur (93).

Methods for separation of simple sugars are generally applicable for oligosaccharides. For separation of small quantities, paper chromatography is an excellent technique (16, 18, 28, 38, 60, 87, 88, 89). Non-reducing oligosaccharides may be detected on the paper by the procedure of Partridge (88), or of Buchanan, Deckker and Long (35). Separation of closely related compounds by paper chromatography is not always possible. Alternative techniques used

on such occasions are electrophoresis in borate buffers (50, 51), and separation from carbon-celite columns (111).

Separation of larger quantities of oligosaccharide has been carried out on cellulose columns (11). The resolution is, however, no better than on paper. A better procedure is the use of carbon-celite columns (111) combined with the gradient elution technique of Alm, et al (3, 4). Dyes may be used to indicate conveniently the position of sugars on the column (62).

A variety of procedures are available for the identification of oligosaccharides. Thus, when standard compounds are available, R_f values may be compared (89). Characteristics of a large number of oligosaccharides have been listed by Whistler and McGilvray (112). Crystalline materials may be compared on a basis of physical measurements. Molecular weights of very small quantities of oligosaccharides may be determined by the "isothermal distillation" technique (34) or estimated from plasmolysis determinations on plant cells (34). Partial and complete hydrolysis with acid and enzyme is perhaps the most valuable technique for evaluating oligosaccharide structure (2, 11, 15, 22, 25, 76, 78). The presence of aldoses in the hydrolytic products may be determined by hypoiodite oxidation (81), and of fructose by treatment with cobaltous chloride and ammonia (37). Separations of hydrolytic products may be accomplished

through osazone formation (85), although chromatographic techniques are generally preferable. Micro-determinations of reducing sugars may be done conveniently by the method of Nelson (84) or of Wagner (106).

A combination of fructose glucose ratios and R_f values has been used for estimating molecular size of glucofructans (114). The correlation of carbohydrate structure with paper chromatogram mobility has been of value in studies of polymeric homologous series (48).

A direct test for the presence of a sucrose linkage in oligosaccharides is available in the Rabin diazouracil reaction (98, 99). Levi and Purves have shown that this test is inoperative when substitution exists in the fructose ring (73).

The structure of original reference compounds is determined usually by identifying the products of hydrolysis of the methylated compound. Improved methods for separation of methylated products are available in paper and column chromatography (32, 48, 64, 74). Structural features, such as chain length or differentiating 1-4 or 1-6 linked hexose units, may be more conveniently determined by periodate oxidation (8, 31, 53, 33, 97). Use of milligram samples separated by paper chromatography is possible by the Warburg respirometer procedure of Perlin (91, 92).

Wheat Leaf Invertase

Although invertase is associated with the synthesis of glucofructans, only the sucrose hydrolysing activity of this enzyme has been studied in detail in the wheat plant, and this only in the leaves.

Invertase, or B-fructofuranosidase, attacks sugars containing fructose in the B configuration and the 2-5 oxygen ring (furanose). The enzyme is therefore not entirely limited to sucrose as substrate, but can attack in addition raffinose, gentianose, stachyose, verbascose and hesperonal. The rate of sucrose inversion, however, greatly exceeds the rate of attack on other sugars (1). The classical Michaelis and Menton equation was derived from studies on this enzyme (79).

Invertase is of general distribution in microorganisms and higher plants. However, it is not necessarily distributed uniformly throughout plant tissues. Thus, invertase is present in leaves of sugar beet (*Beta vulgaris* L.), but lacking in the root (2). Similarily, invertase is present in the leaves of broad bean (*Vicia faba* L.) but absent from cotyledons (2). Archbold (5) found invertase present in leaves of barley, but there is no information on its presence in stems.

The kinetics of sucrose hydrolysis by wheat leaf invertase has been reported on by Roberts (103) and by

Ward (107). Apart from a much lower Michaelis constant, the kinetics resemble those of yeast invertase (43, 107). The optimum pH was around 4.5, and the optimum temperature above 40° C., at least for very short exposures. A temperature of 27.5° C. and a substrate concentration of 1.6% was found suitable by Ward for quantitative determinations of invertase. Roberts (103), however, greatly increased the linear portion of the progress curve by increasing the sucrose concentration to around 4%. Both Ward and Roberts found that purification procedures considerably inactivated the invertase from wheat leaves. Consequently, an untreated juice obtained by grinding fresh leaves was utilized as the source of active enzyme.

Synthesis of at least one glucofructan through transfructosidase activity of wheat leaf invertase is known to occur (2). The kinetics of the enzyme activity have not been studied. However, transfructosidase kinetics of invertases from other sources suggest that the enzyme functions over a broad range of conditions. Thus, Edelman (41) found that glucofructans were formed by yeast invertase over a substrate concentration of 1 to 60%, and over a pH range of 3 to 9 with citrate, acetate, phosphate and veronal buffers.

Bhatia, et al (23) found an optimum sucrose concentration of 30% for transfructosidase activity of extracts from

Agave. With this substrate concentration, a linear progress curve was obtained between 6 and 52 hours. The linear portion of the curve did not, however, project through the origin. Bhatia, et al (23) found the optimum pH at 5.6-5.8 from Agave transfructosidase, with an optimum temperature of 37° C. A temperature of 55° C. inactivated the enzyme.

General methods of measuring transglycosydase activity of invertases has been discussed by Bacon (12). The most satisfactory method of separating the oligosaccharides from the enzyme digest is by paper chromatography or on a carbon column.

EXPERIMENTAL

Structure of the Wheat Stem

Before examining wheat stem glucofructans, a brief description is presented of the structure of the wheat stem, following Esau (42), Percival (90), and Robbins (101).

The wheat stem is of the general grass type, the cylindrical culms being divided into sections or internodes. In young stems, both nodes and internodes are solid. As the stem enlarges, the pith generally ruptures above and below the nodes, forming a pith cavity surrounded by colourless ground parenchyma. The cells forming this ground parenchyma soon die, and lose their cell contents.

Generally, culms of wheat have six internodes, the sixth bearing the spike. The first and second internode remain several millimeters in length, while the sixth accounts for a major part of the height of the plant. Lower internodes, as well as much of the upper internodes, are covered by a leaf sheath. Photosynthesis is carried on actively by chlorenchyma of the stem and leaf sheath. In fact, wheat grain will mature even when leaf blades are removed (90).

In young plants, cell division occurs throughout the length of the internode. Later, meristematic activity gradually becomes confined to the internode base and finally disappears. Intercallary meristems are not completely

undifferentiated, but contain vascular and supporting cells allowing continuity of these tissues throughout the length of the stem.

In structure, the wheat culm comprises 5 tissues: the hypoderm, or zone of mechanical tissue beneath the epidermis; green assimilating parenchyma; colourless ground parenchyma; the vascular bundles. The distribution of these tissues is shown diagramatically in Fig. I.

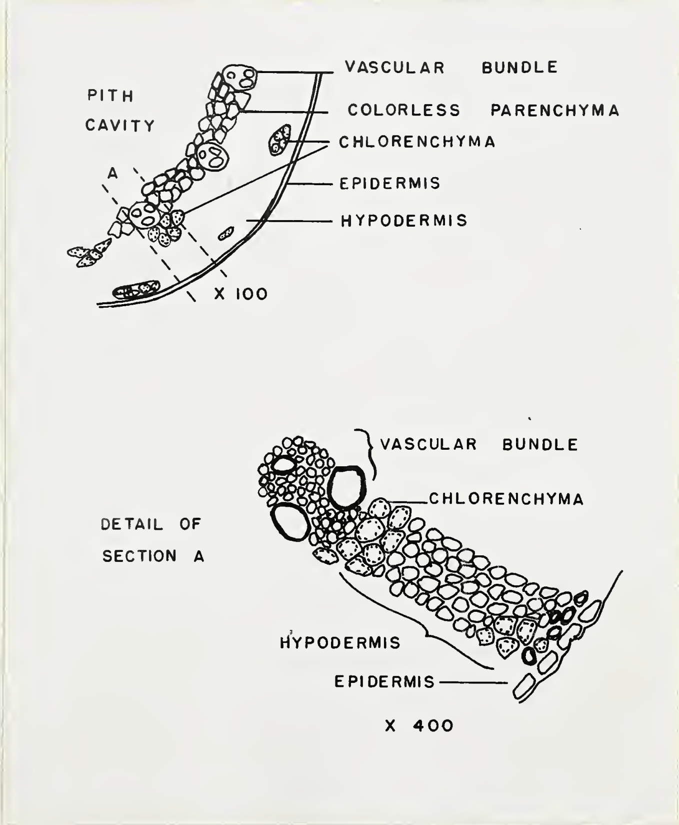


Fig. I. Cross section of stem of _Triticum vulgare_ showing distribution of tissues.

Stems of Golden Ball, a variety of Triticum durum Desf. utilized extensively in the present work, remain solid throughout all stages of growth and growing conditions (95). This wheat is of particular interest since its stems are highly resistant to attack by the wheat stem sawfly (Cephus cinctus Nort.) (96).

Glucofructans from Wheat Stems

Alcohol Soluble Glucofructans

A series of oligosaccharides isolated from wheat stem was examined first by paper chromatography. This procedure necessitated extraction of sugars from the stems, followed by purification and concentration of the extract. The methods used were adapted from Laidlaw and Reid (70), and Wylam (119). An outline of the experimental procedure is given below. The wheat selected for this particular study was Golden Ball.

Preparation of plant samples

Samples of field grown wheat were taken at the milk stage when oligosaccharides were at peak concentration. The plants were cut at ground level, the spike was removed at the first node, and all leaf coverings stripped from the stem. The stems were then cut to a convenient length and immersed in boiling 87% ethanol and boiled for three minutes. This alcohol heat treatment served the double purpose of inactivating enzymes and preserving the material during

storage. Stems were stored in this form at -5° C. prior to further processing.

Preparation of stem extracts

Following decantation of alcohol, the stems were macerated in a Waring blendor for three minutes. The plant debris was then transferred to a Soxhlet and extracted with 80% ethanol for three hours. Chromatographic examinations showed that extraction of alcohol soluble carbohydrates was then complete.

The alcoholic extract was evaporated to a syrup under vacuum at 39° C., and the residue taken up with water. Clarification was accomplished with equivalent quantities of barium hydroxide and aluminum sulphate as described by Browne and Zerban (34). This procedure leaves a minimum of ionic material in solution. Deionization was accomplished by passing the filtrate through the ion exchange resins, Amberlite IRC-50 and IR-4B. Specific resistance of deionized solutions was kept above 35,000 ohms to obtain chromatographic spots without tails.

Chromatographic procedure

Chromatographic separation of oligosaccharides was accomplished by the paper partition procedure of Partridge (87, 88) using butanol, acetic acid, water solvent (4:1:5) and Whatman No. 4 paper. As control sugars, spots of 1.0%

solutions of raffinose, and sucrose were run on each paper. Spots were applied in a volume of .002 ml with a Gilmont micro-burette. Development times varied from sixteen hours for separation of oligosaccharides from the hexose sugars, up to 12 days for complete separation of oligosaccharides from each other.

Chromatograms were sprayed with a 14:1 mixture of naphthoresorcinol trichloroacetic acid reagent of Partridge (87), and benzidine reagent of Horrocks (61). This mixture was equally sensitive to aldo and keto sugars, and, providing the papers were subsequently washed in water, produced a chromatogram reasonably stable for several months. The sprayed papers were heated for 3 minutes at 120° C.. Fructose appeared bright yellow, glucose dark brown, while sucrose, raffinose and oligosaccharides were of intermediate colour. Reducing activity was examined with the silver nitrate-ammonia spray of Partridge.

When carbohydrates were eluted from the developed chromatogram, the carbohydrate zones were estimated from sprayed strips removed previously from the edge of the paper by the method of Flood, Hirst and Jones (46). Elution was carried out at room temperature by the method of Laidlaw and Reid (70). The milligram quantities of oligosaccharides required for hydrolytic and specific colour tests were obtained by running preparations on Whatman 3MM. paper. In

this procedure 10 to 24 spots were applied at intervals of 1/4 inch along the zero line of the chromatogram, individual spot application being increased to .008 ml.

With the slow moving oligosaccharides the solvent front had progressed well off the end of the paper before separation was complete. Consequently, all mobilities were determined against raffinose as a standard.

Permanent records were made by preparing tracings of the freshly prepared chromatographs. Photographs of the tracings are presented throughout this work.

Oligosaccharide mobilities

The relative rates of movement of the oligosaccharides from the stem of Golden Ball are illustrated in the chromatograms in Fig. 2. This illustrates the complete separation of fructose, glucose and sucrose, while the non-reducing oligosaccharide fraction is just commencing to migrate.

Similar chromatograms developed for 6 and 12 days are shown in Fig. 3, which indicates that at 6 days two oligosaccharides have progressed beyond raffinose, while fractions III and IV show elongated spots suggesting mixtures. At 12 days, raffinose and the first two oligosaccharides have moved off the end of the paper; fraction III has split into IIIA and IIIB; fraction IV into IVA and IVB, while fraction V has separated from the spot trailing from the origin. No attempt was made to fractionate further the

material at the origin, interest being confined to the seven separated oligosaccharides.

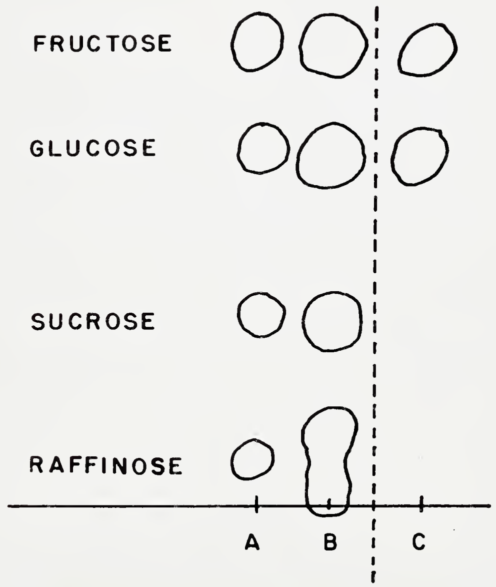


Fig. 2. Chromatogram of Golden Ball stem extract developed 24 hours. A, control sugars; B and C, stem extract. A and B, sprayed with naphthoresorcinol-bendizine; C, sprayed with alkaline silver nitrate.

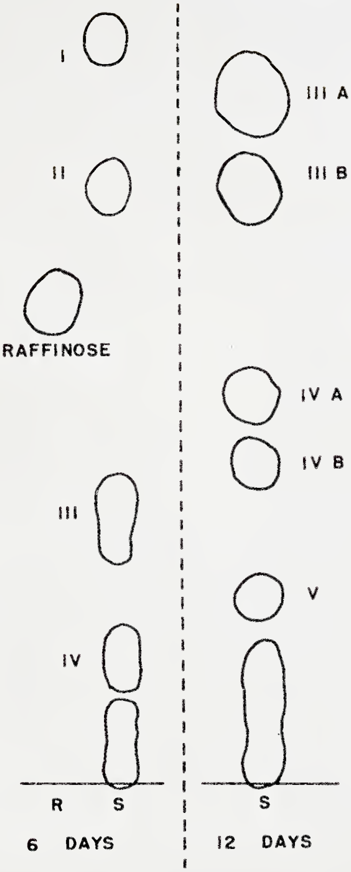


Fig. 3. Chromatograms of Golden Ball stem extract developed 6 and 12 days. Oligosaccharides are indicated by Roman numerals; R: control sugar raffinose; S: stem extract.

The relative rates of movement of the oligosaccharides upon the chromatogram were determined using raffinose as a standard. The distance of the compound from the origin, divided by the distance moved by raffinose on the same chromatograph, was recorded as the R_R value. These values are listed in Table I.

TABLE I

R_R VALUES OF WHEAT STEM OLIGOSACCHARIDES

Oligosaccharide	Distance, cm.*	R_R value
Reffinose	38.9	1.0
I	58. **	1.49
II	39.5	1.02
IIIA	25.7	.66
IIIB	21.6	.57
IVA	13.8	.35
IVB	11.3	.29
V	6.3	.16

* Mean calculated from 11 chromatograms run for 12 days; Whatman No. 4 paper.

** Extrapolated from chromatograms run for 7 days.

Fructose-glucose ratio of oligosaccharides

Previous studies indicated that fructose and glucose were the only sugars produced by hydrolysis of wheat stem

oligosaccharides (40). The relative amount of fructose and glucose in the individual oligosaccharides was, therefore, estimated. For this purpose, chromatograms were run on Whatman 3MM paper, and the separated compounds eluted in a total volume of 2 ml. at room temperature by the method of Laidlaw and Reid (69). The analytical procedure entailed a total sugar determination, plus a specific analysis for fructose. Glucose was then determined by difference.

Total sugar was determined by the micro-colorimetric method of Nelson (84), while fructose was determined by the resorcinol method of Roe (100), with heating time increased to 15 minutes as recommended by Roberts (102).

Since Nelson's procedure is applicable only to reducing sugars, a preliminary hydrolysis of the oligosaccharides was necessary in determining total sugars. This was accomplished by heating the oligosaccharides for 30 minutes in the presence of 1.4N hydrochloric acid, followed by precise neutralization of the acid with sodium hydroxide. Some decomposition of the reducing sugars apparently occurs under this treatment. It was found essential, therefore, to include glucose and fructose controls in the hydrolysis procedure. An outline determination in triplicate of the fructose-glucose ratio of sucrose by this procedure is enumerated below.

	Optical density at 540 Mu		
Procedure	fructose*	glucose*	hydrolysed sucrose
Resorcinol (fructose)	1. - 135 2. - 132 3. - 133 400		1. - 141 2. - 139 3. - 145 425
Arseno-molybdate (total sugars)	1. - 84 2. - 82 3. - 84 250	1. - 90 2. - 87 3. - 89 266	1. - 185 2. - 182 3. - 178 545

* 30.8 mg. per liter

Calculation of the fructose-glucose ratio of sucrose from these data was carried out as follows:

From resorcinol determinations:

30.8 mg. fructose = optical density of 400

$\therefore$ fructose content of sucrose of density 425 =

$$\frac{425}{400} \times 30.8 = 32.8 \text{ mg. fructose.}$$

From arseno-molybdate determinations:

30.8 mg. fructose = density 250

$$\therefore 32.8 \text{ mg. fructose} = \frac{32.8}{30.8} \times 250 = \text{density of } 266.$$

Total density of sucrose = 545

less density for fructose = 266

279 = density for glucose.

Since density of 266 = 30.8 mg. glucose,

$$\therefore \text{density of } 279 = \frac{279}{266} \times 30.8 \text{ mg.} = 32.3 \text{ mg. glucose}$$

$$\therefore \text{fructose to glucose ratio} = \frac{32.8 \text{ mg. fructose}}{32.3 \text{ mg. glucose}} = \frac{1.015}{1},$$

$$\text{Theoretical ratio} \qquad = \frac{1.00}{1.00}$$

Determinations of the fructose-glucose ratios of wheat stem oligosaccharides produced the results shown in Table II.

TABLE II

FRUCTOSE-GLUCOSE RATIO OF WHEAT STEM OLIGOSACCHARIDES

Oligosaccharide	Fructose-glucose	assumed theoretical ratio	assumed D.P.*
I	2.03/1; 1.78/1; 1.97/1.	2/1	3
II	1.8/1; 1.88/1; 2.23/1.	2/1	3
IIIA	3.02/1; 2.8/1; 2.96/1.	3/1	4
IIIB	3.37/1; 2.9/1; 2.7/1.	3/1	4
IVA	3.7/1; 3.72/1;	4/1	5
IVB	3.84/1; 3.46/1.	4/1	5
V	4.7/1	5/1	6

* D.P. = degree of polymerization

The assumed theoretical ratios of fructose and glucose, and hence the degree of polymerization, were estimated from the analytical results, and from mobilities of the

respective compounds upon chromatograms. Oligosaccharide I and II, for example, produced an approximate fructose-glucose ratio of 2/1. The proximity of both these compounds to the trisaccharide raffinose on the chromatogram indicates a degree of polymerization of three.

Plotting the degree of polymerization of the oligosaccharides against their R_R values on a semi-log. scale produced the results shown in Fig. 4.

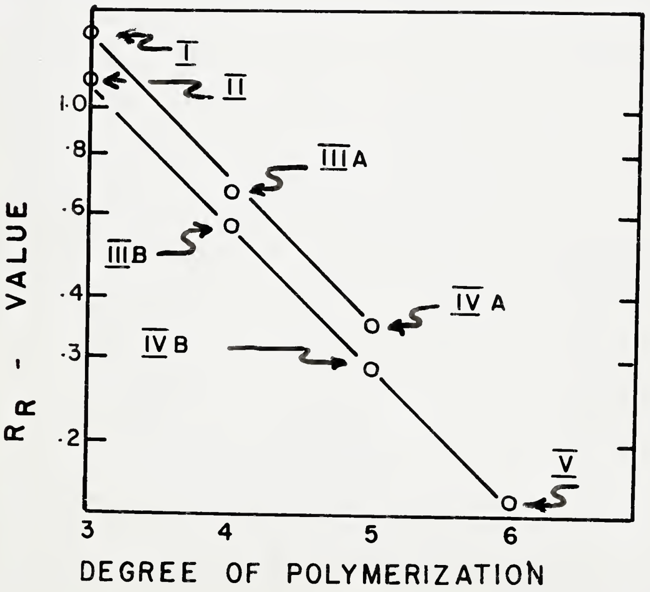


Fig. 4. The degree of polymerization of wheat stem oligosaccharides plotted against the log. of chromatogram mobilities (R_R values).

It has been shown by French and Wilde (48) that a

linear relationship between the log. of mobility and degree of polymerization indicated an homologous series among certain naturally occurring oligosaccharides. The linear slopes of the two parallel curves shown in Fig. 4 suggests the presence of two homologous series of glucofructans, both series terminating in trisaccharides.

It seemed, therefore, that the most fruitful line of attack in structural studies of the glucofructans would be an examination of the two trisaccharides. Consequently, an attempt was made to identify the products produced by partial hydrolysis of these compounds.

Partial hydrolysis of fraction II (present in highest concentration) was first attempted with maltase. The enzyme, obtained from Nutritional Biochemicals Corporation, proved to be badly contaminated with oligosaccharides. These were removed by precipitation of the enzyme at 0° C. with ethanol.

However, partial hydrolysis of fraction II with this enzyme was unrewarding, as fructose and glucose were the only products which could be identified. Dilute sulphuric and hydrochloric acid (0.1N) produced similar results.

Partial hydrolysis with acetic acid, carried out under the following conditions, was more successful. Equal quantities of oligosaccharide fraction II and 0.2N acetic acid were added to a micro tube and heated for 2 minutes in a boiling water bath, and then cooled. The mixture was then

spotted directly on Whatman No. 1 paper. Spots of fructose, glucose and sucrose and untreated fraction II were included as controls. Overnight development produced the results shown in Fig. 5.

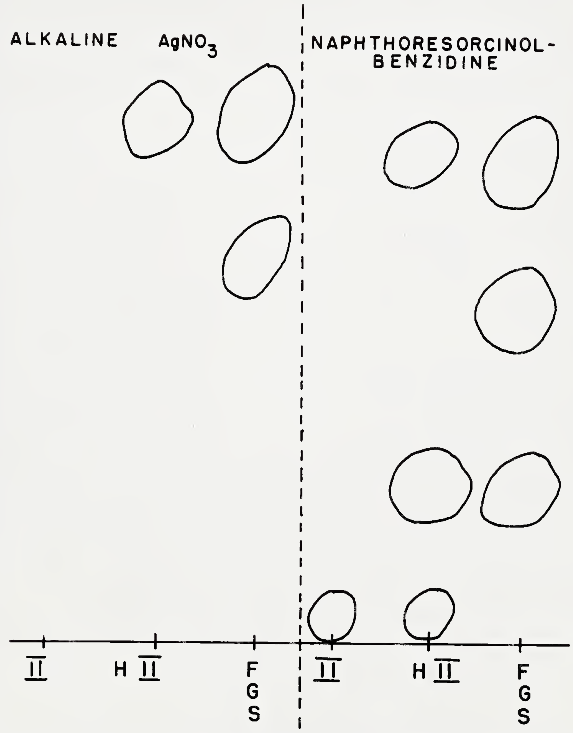


Fig. 5. Partial hydrolysis of oligosaccharide fraction II with 0.1N acetic acid. Reducing compounds are shown on the chromatogram sprayed with silver nitrate.

F G S : fructose, glucose and sucrose;
II : untreated oligosaccharide;
HII : partially hydrolysed oligosaccharide.

Fig. 5 shows that partial hydrolysis of fraction II produced a reducing spot corresponding to fructose, and a non-reducing spot corresponding to sucrose. To confirm the presence of sucrose, a similar chromatogram was prepared and the position of the supposed sucrose spot estimated by removal and spraying of an edge strip. A drop of invertase solution was then placed over the sucrose spot, and the paper placed in a moist chamber at room temperature for 2 hours. Subsequent development of the chromatogram at right angles to the original run revealed spots of fructose and glucose of apparent equal concentration.

Fraction I was then treated similarly with acetic acid, and the resulting solution chromatographed. The results are shown in Fig. 6. It may be seen from Fig. 6 that the products of partial hydrolysis are fructose, and sucrose. Following direct treatment of the sucrose spot with invertase two similar sized spots of fructose and glucose were formed upon re-development of the chromatogram.

Based upon the above results, plus the ease of hydrolysis, it was tentatively concluded that both oligsaccharide I and II consist of a fructo-furanose unit linked through a β glycoside linkage to a sucrose residue.

In the sucrose molecule there are eight possible points for glycosidic attachment of an additional fructose unit. Three of these points are primary alcoholic groups,

while five are secondary groups.

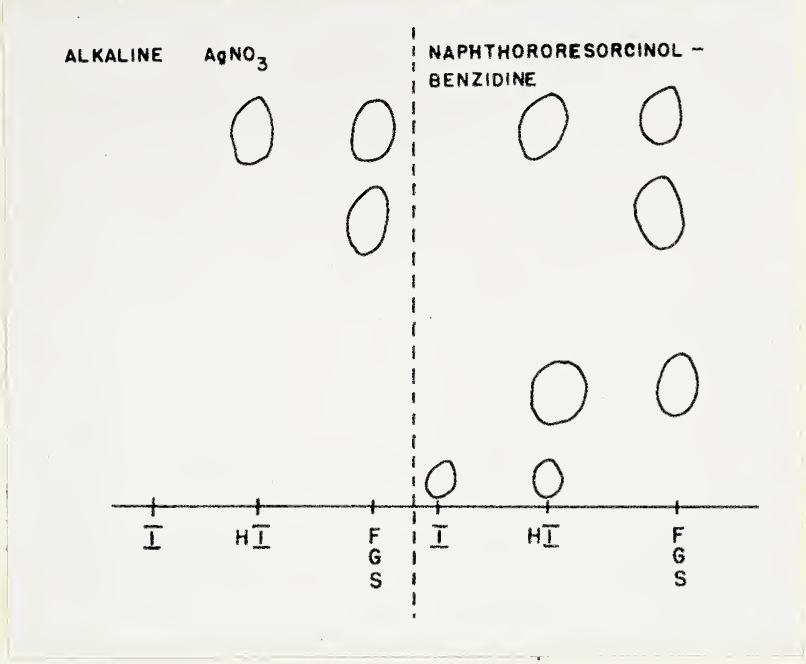


Fig. 6. Partial hydrolysis of oligosaccharide I with acetic acid.

F G S : fructose, glucose and sucrose;
I : untreated oligosaccharide fraction I;
HI : partially hydrolysed oligosaccharide I.

One method of limiting the possibilities of attachment fortunately exists in Rabin's diazo-uracil reaction. This reaction gives a positive test for the presence of a sucrose like bond, only when the fructose unit remains unsubstituted (73).

Because the sensitivity of the Rabin reaction was low, (requiring 40-50 mg. of sucrose), eluates from chromatograms

could not be tested directly. To overcome this difficulty, two expedients were necessary. First, larger quantities of oligosaccharides were obtained by fractionation of stem extracts on carbon columns. Secondly, the sensitivity of the Rabin test was increased by altering the conditions of reaction.

Separation of oligosaccharides was carried out on a carbon-celite column by the method of Whistler and Durso (111), coupled with the gradient elution procedure of Alm, et al (3, 4). Briefly, the procedure was as follows:

Equal quantities of activated charcoal (B.D.H.) and celite (Johns-Manville) were mixed to form a slurry with water. An equal volume of concentrated hydrochloric acid was added, and the mixture stirred from time to time over a period of several hours. The mixture was then filtered and washed with water until no test for chloride could be obtained in the filtrate with silver nitrate. The slurry was then poured into a 40 x 600 mm. column, and 4 ml. of wheat stem sugar extract in the form of a syrup added (approximate concentration, 50%). Elution was done from a reservoir of 10% ethanol, the latter being connected by a siphon to a supply of 25% ethanol. In this way a gradually increasing concentration of ethanol was obtained. As shown by Alm, et al (3, 4), this procedure increases resolution of a chromatographic column since band spreading is

counteracted by an increased rate of elution of the band tails.

The eluate was collected in 10 gm. samples in a Shandon gravity-type fraction collector. Monitoring of the samples was accomplished by spotting filtrate from each tube on filter paper, followed by treatment with naphthoresorcinol benzidene sugar reagent in the usual way. Relative concontrations of sugars in the separated fractions were estimated by measuring spot density on the paper with a photovolt densitometer (77). The results of the densitometer readings are shown in Fig. 7.

The order of elution of sugars from the carbon column was determined by paper chromatography, samples being taken from tubes of peak spot density. An unresolved mixture of fructose and glucose appeared first from the column, followed by sucrose and the higher oligosaccharides. As seen in Fig. 8, the order of emergence of the trisaccharides was reversed from that of paper separation.

Judging from the density of chromatographic spots, it was estimated that the concentration of sugar in the tubes at peak density was between one to two per cent, giving a total of 10 to 20 mg. per tube. The tubes showing maximum concentration of trisaccharide were, therefore, evaporated to dryness under vacuum, and the residue taken up in 3-6 drops of water, A modified Rabin reaction was then carried

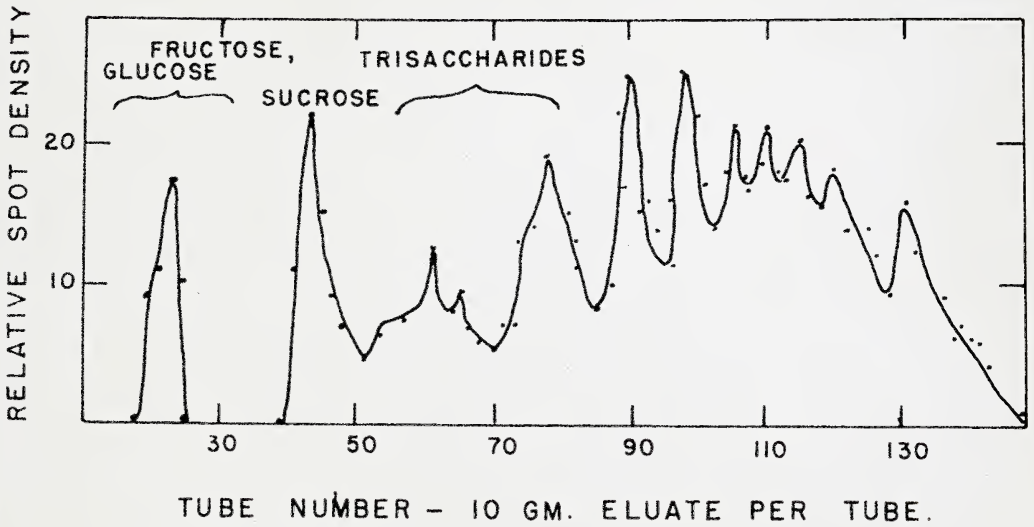


Fig. 7. Separation of oligosaccharides from wheat stems on carbon column, using gradient elution with ethanol. For details see text.

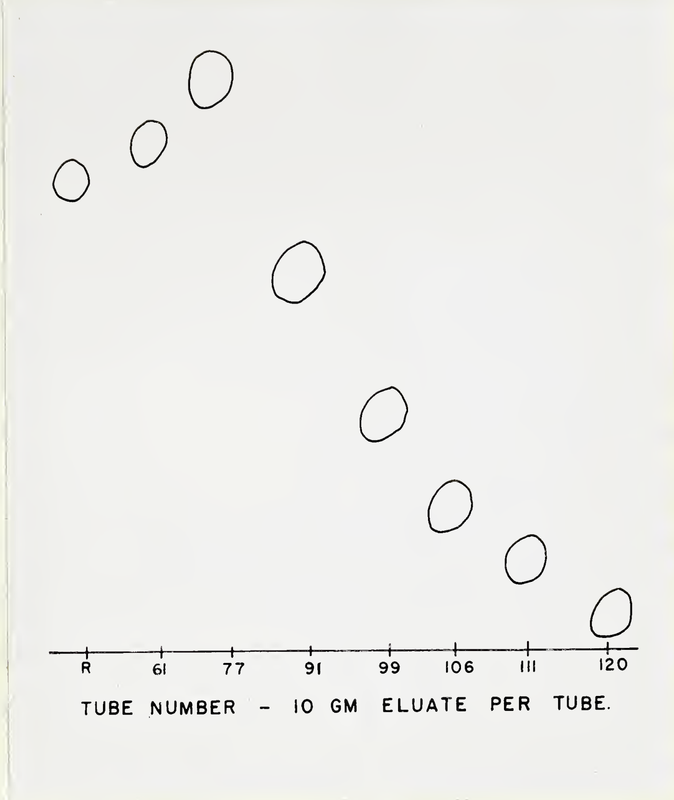


Fig. 8. Chromatogram produced from oligosaccharides separated on carbon-celite column. Chromatogram ran for 5 days on Whatman No. 4 paper with butanol-acetic acid solvent. R: raffinose.

out on this material, as follows:

One drop of sugar solution was placed in a 3 ml. micro centrifuge tube containing one drop of 0.1N sodium hydroxide. Just sufficient powdered diazo-uracil was added to produce a faint pink, which faded within 2 to 3 minutes. The tube was kept for 5 minutes at 10° C. and then one small crystal of magnesium sulphate was added. A definite blue

coloration, turning green with addition of magnesium, was given by 0.1 mg. of sucrose.

Similar tests carried out on 3 to 6 mg. samples of the two trisaccharides gave negative results. This was considered presumptive evidence that the second fructose unit in both trisaccharides was linked to sucrose through fructose, rather than through the glucose unit.

Of the eight possible non-reducing glycosides formed by addition of fructose to sucrose, three have just recently been characterized. These are the three primary alcohol substitution products 6^F-B-fructosylsucrose, 1^F-B-fructosylsucrose, and 6^G-B-fructosylsucrose. All are formed from sucrose by yeast invertase (11).

The chromatographic mobility of the above three compounds was compared with that of the wheat stem trisaccharides. For this purpose, a yeast invertase digest was prepared following Bacon's procedure (12), and examined periodically by paper chromatography. The digest consisted of 1.0 ml. of 25% sucrose, 0.25 ml. of phosphate-citrate buffer of pH 5.0, and one drop of yeast invertase solution (Bios Laboratories). Incubation was at room temperature. The chromatogram produced after 30 minutes incubation is shown in Fig. 9.

The results of Fig. 9 show close agreement between the mobilities of the two trisaccharides from wheat stems with

those present in the yeast invertase digest.

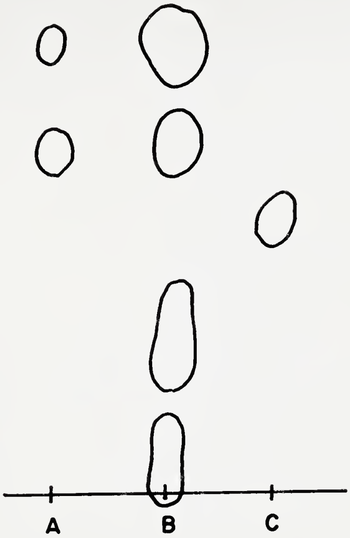


Fig. 9. Chromatographic mobility of trisaccharides from yeast invertase digest compared to those from wheat stems. A: yeast invertase-sucrose; B: wheat stem extract; C: control trisaccharide, raffinose. The upper spot from the invertase digest consists of 1^F-B-fructosylsucrose and 6^G-B-fructosylsucrose, the lower of 6^F-B-fructosylsucrose.

Chromatogram developed 5 days with butanol-acetic acid-water, and sprayed with naphthoresorcinol-benzidine.

From these results the slower moving trisaccharide from wheat stems was tentatively identified as F^6-B-fructosylsucrose. The faster moving trisaccharide, because of a negative Rabin reaction, was tentatively identified as

F^1-B-fructosylsucrose, rather than G^6-B-fructosylsucrose or a mixture of these two.

Water Soluble Glucofructans, (Insoluble In 80% Alcohol)

Following extraction of wheat stems with 80% ethanol, additional carbohydrate material was obtained by extraction with cold water. This material, when chromatographed, revealed one major spot of very low mobility plus traces of oligosaccharides similar to those present in alcoholic extracts.

Trace contaminants were removed by precipitating the glucofructan several times from 75% ethanol. The precipitate was separated by centrifugation, washed with 75% ethanol, and finally dried over sulphuric acid. The product, a white, amorphous material, was examined by a combination of partial hydrolysis and chromatography, in a manner similar to that described previously. The results of partial and complete hydrolysis with invertase are shown in Fig. 10. This indicates that the glucofructan is hydrolysed completely, forming fructose and a trace of glucose. A negative silver nitrate spray reaction implies that both the intact glucofructan, as well as the partially hydrolysed residue, are non-reducing.

Chromatograms showing the results of partial and complete hydrolysis with acetic acid are shown in Fig. 11.

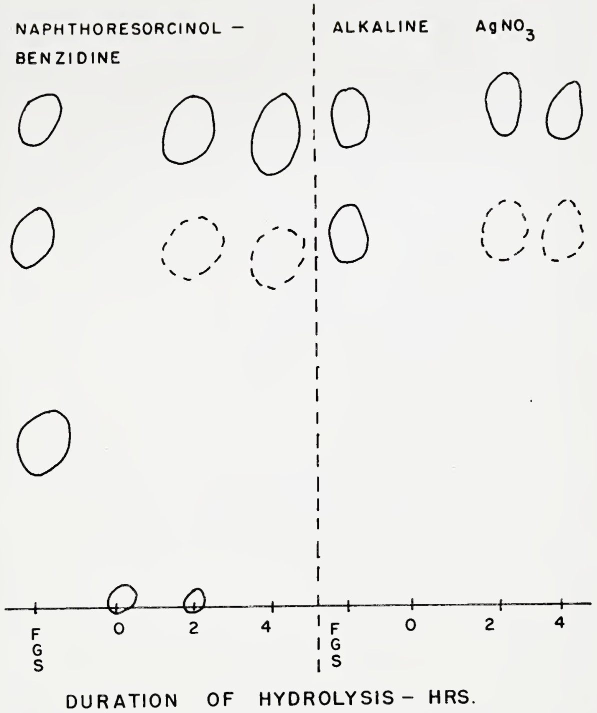


Fig. 10. Partial and complete hydrolysis of water soluble glucofructan with invertase. Dotted lines represent faint chromatographic spots.
F G S : fructose, glucose and sucrose.

Fig. 11 illustrates a number of distinctive features of acid hydrolysis of the water soluble glucofructan. First, a spot (presumably a disaccharide) of the same R_F as sucrose was produced. As shown by the reaction to alkaline silver nitrate, this compound possessed reducing power. Secondly, the residue remaining at the origin after partial

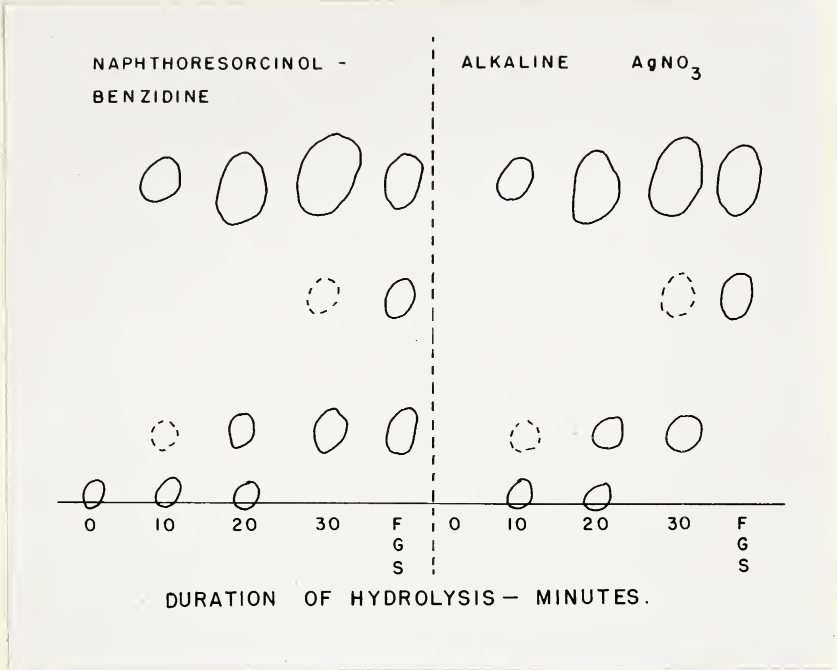

NAPHTHORESORCINOL - BENZIDINE
ALKALINE AgNO3
0
10
20
30
F G S
0
10
20
30
F G S
DURATION OF HYDROLYSIS — MINUTES.

Fig. 11. Partial and complete hydrolysis of water soluble glucofructan with acetic acid. Dotted lines represent faint chromatographic spots.
F G S : fructose, glucose and sucrose.

hydrolysis, also possessed reducing power.

Hydrolysis of the reducing disaccharide directly on the paper was carried out with invertase. Subsequent redevelopment produced similar sized spots corresponding to fructose and glucose. These results suggested that the unknown disaccharide might be turanose. The partially hydrolysed glucofructan was, therefore, spotted on a fresh chromatogram along with turanose. Development of the

chromatogram for 60 hours produced the results shown in Fig. 12.

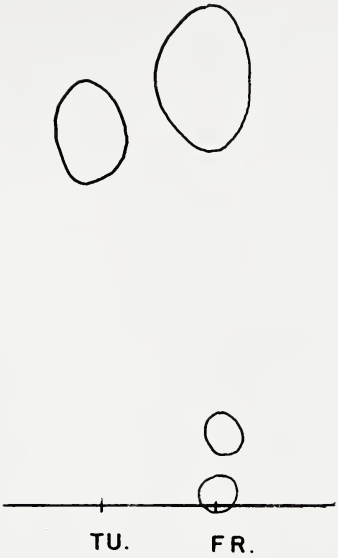


Fig. 12. Comparison of turanose and the reducing disaccharide formed by partial hydrolysis of water soluble glucofructan.

The figure illustrates that the reducing disaccharide produced by partial hydrolysis of the water soluble glucofructan is not turanose. The chromatogram shows also that the residue remaining after partial hydrolysis has separated into two reducing fractions.

From the experimental results, certain conclusions were drawn regarding the nature of the alcohol insoluble

glucofructan. First, water solubility limits the molecular size to around twelve hexose units. Consequently, the fructose-glucose ratio of around 9-10 to 1.0, suggests only one unit of glucose per molecule. Secondly, from the ease of hydrolysis with invertase, fructose units are almost certainly in furanose form, and joined by beta linkage. Thirdly, since the glucofructan is non-reducing, all anomeric carbons are involved in linkage. This can occur only (a) if the terminal units of the sugar chain are joined to form a ring; (b) a double anomeric carbon linkage (such as in sucrose) occurs in the chain.

Ring formation may be ruled out, since a reducing residue would remain following partial hydrolysis with invertase, disagreeing with the experimental results. It follows then that the alcohol insoluble glucofructan possesses a double anomeric carbon link. Since linkage of two fructofuranose units through anomeric carbons has not been described, it seems probable that the linkage involved is a sucrose bond.

Such a compound may be represented diagramatically as follows, where sucrose linkage is shown as a high energy bond:

$$\text{F-F-F-F-F} \sim \text{G-F-F-F-F}$$

In a compound of this nature, the sucrose link is protected from enzyme action by substitution in both the

fructose and glucose ring. Consequently, invertase would be expected to peel off fructose units from either end, always leaving a non-reducing residue. This type of breakdown is substantiated by the experimental results.

The sequence of events following acetic acid hydrolysis is more difficult to evaluate. From the density and mobility of chromatographic spots, seemingly the compound is broken fairly early into two reducing residues of almost equal size. This would result only if the break occurred at the sucrose bond. Further hydrolysis would produce free fructose, and finally leave the presumably more resistant reducing disaccharide, -G-F.

Biosynthesis of Glucofructans

Following the examination of oligosaccharides, an attempt was made to identify the enzyme system synthesizing these compounds in wheat stems.

Seemingly, at least the higher members of glucofructans from wheat stems must be formed *in situ*, since translocation of such large molecules through phloem tissue is dubious. This conclusion, however, is by no means certain. For instance, it is known that at the stage of growth when glucofructans first appear in wheat stems, similar compounds also appear in the leaves (75). Furthermore, although sugar is preferentially translocated as sucrose, Zimmermann (120,

121) has shown that the trisaccharide raffinose, and the tetrasaccharide stachyose can be translocated, at least through the sieve tubes of trees. Thus, the possibility that glucofructans are formed in the wheat leaves and translocated to the stems cannot be ruled out. On the other hand, the similarity of photosynthetic tissues of leaves and stems suggests that possibly glucofructans arise from both these sources.

It was decided, therefore, to determine first whether the source of glucofructans was the wheat stem, the wheat leaf, or both tissues. Since the only known system synthesizing glucofructans in higher plants is that of invertase, the activity of this enzyme in extracts from leaves and stems was first examined.

Transfructosidase Activity of Leaf and Stem Extracts

Characterization of the oligosaccharides formed by leaf and stem invertase was determined chromatographically. Leaf and stem extracts were prepared by grinding fresh tissue with an equal weight of phosphate-citrate buffer of pH 4.7 in a mortar with sand. The juice was then expressed at 5000 lb. in a Carver hydraulic press. An equal quantity of 20% sucrose solution was added to the juice, and the digests incubated for 24 hours at 28° C. in the presence of a few drops of toluene. Periodically, samples were

withdrawn and heated 2 minutes in a boiling water bath to inactivate the enzyme. The digests were then examined for sugars by paper chromatography. The results obtained following 24 hours incubation are shown in Fig. 13.

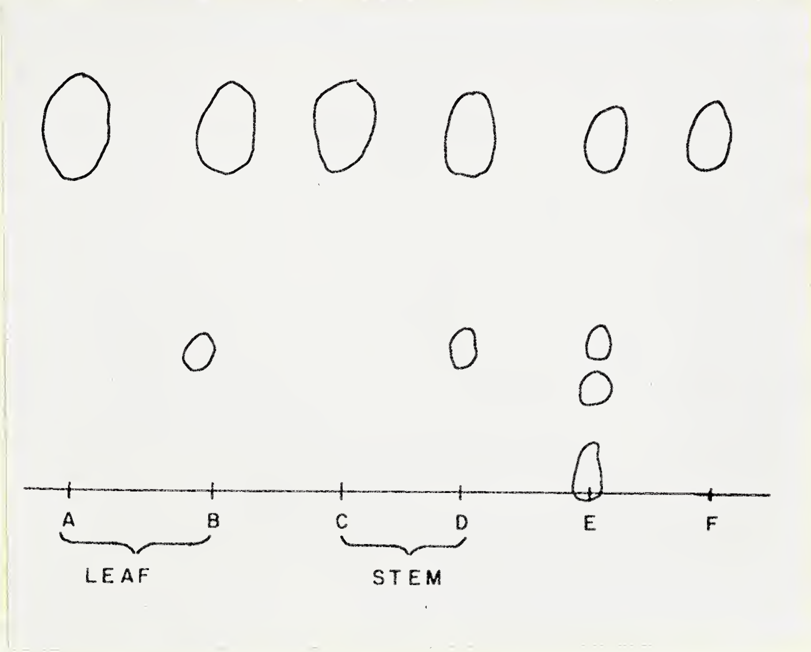


Fig. 13. Synthesis of oligosaccharide from sucrose by extracts of leaves and stems. A and B, heated and active leaf extracts; C and D, heated and active stem extracts; E, concentrated sugar extract from wheat stems. F, sucrose. Fructose and glucose have moved beyond the borders of the chromatogram.

Fig. 13 shows that both leaf and stem extract formed only one oligosaccharide. This compound possessed an R_f value equivalent to that of the most mobile glucofructan

from wheat stems, and gave an exactly similar colour reaction with naphthoresorcinol and benzidine sprays. Further, the oligosaccharide was non-reducing to ammoniacal silver nitrate, and was hydrolysed completely by invertase to fructose and glucose.

From these results it was concluded that enzyme activity of either stems or leaves could synthesize from sucrose, at least, the lowest member of the glucofructan series. To determine if invertase were indeed the enzyme involved, the correlation between sucrose hydrolysis and oligosaccharide synthesis was examined.

Quantitative Determinations of Invertase and Transfructosidase in leaves and stems

In the previous section it was shown that the transfructosidase activity of invertase was a possible synthetic mechanism for wheat stem oligosaccharides. If only one enzyme is involved, then fructose transfer and sucrose hydrolysis should run parallel. To test this hypothesis, the rates of sucrose hydrolysis and transfructosidase activity were compared in leaves and stems at progressive stages of growth.

Leaf and stem extracts were obtained by grinding tissue with sand, as described previously. The juice was stored overnight at 4° C. under toluene, and then clarified by

centrifuging. Further storage, when necessary, was done at 4° C. under toluene. A gradual drop in activity of the extracts occurred with time. Consequently, sucrose hydrolysis and transfructosidase determinations were always determined on the same sample.

Quantitative enzyme determinations must be carried out under conditions where the enzyme alone limits the rate of reaction (105). To determine the conditions required for analysis of invertase (sucrose hydrolysis) and transfructosidase, preliminary kinetic studies were required. These kinetic studies are described in the following section.

Invertase

(a) Progress curve

To establish an invertase progress curve, the velocity of sucrose hydrolysis was measured over a 2 hour period. Sucrose hydrolysis was determined by measuring the increase in invert sugar by Nelson's colorimetric method (84).

Since chromatographic evidence indicated little difference between the invertase of stems and leaves, the progress curve was prepared using stem enzyme extracts only. Conditions were similar to those established by Ward for analysis of wheat leaf invertase (107). The conditions were as follows:

Enzyme reaction mixtures were prepared by adding 1.0

ml. of enzyme to test tubes containing 0.5 ml. of phosphate-citrate buffer of pH 4.7, and 0.5 ml. 10% sucrose. Controls consisted of similar tubes prepared from enzyme previously heated for 2 minutes in a boiling water bath. Tubes were incubated at 28° C. for periods up to 3 hours. Following incubation, the enzyme was inactivated by heating for 2 minutes in a boiling water bath. The resulting progress curve is shown in Fig. 14.

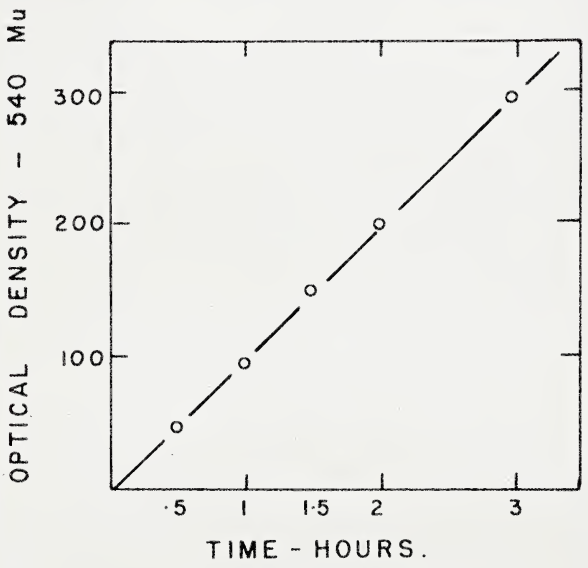


Fig. 14. Progress curve for wheat stem invertase. Optical density of 200 is equivalent to 25 mg. of reducing sugar (as glucose).

Fig. 14 shows that the reaction velocity of invertase under the above conditions possesses a linear relationship with time for at least 3 hours.

(b) Concentration curve.

Enzyme digests were prepared in a similar fashion to those used for the progress curve, except that the enzyme concentration was varied from 0.25 ml. to 1.5 ml., with a total volume of 2.0 ml. An incubation time of 2 hours was selected as lying well within the period of linear response to time. The enzyme concentration curve drawn from the results is presented in Fig. 15.

Fig. 15 shows that invertase activity remains practically linear over the whole concentration range. A concentration of 1 ml. enzyme in a total volume of 2.0 ml. digest was used in all subsequent invertase analysis.

Transfructosidase

The determination of transfructosidase activity was complicated by the analytical procedure. Direct determination of oligosaccharides formed in the reaction mixture is not possible (109). Consequently, the material was separated by paper chromatography, followed by elution and quantitative estimation. The chromatographic procedure utilized was similar to that described previously for determination of hexose ratios of glucofructans.

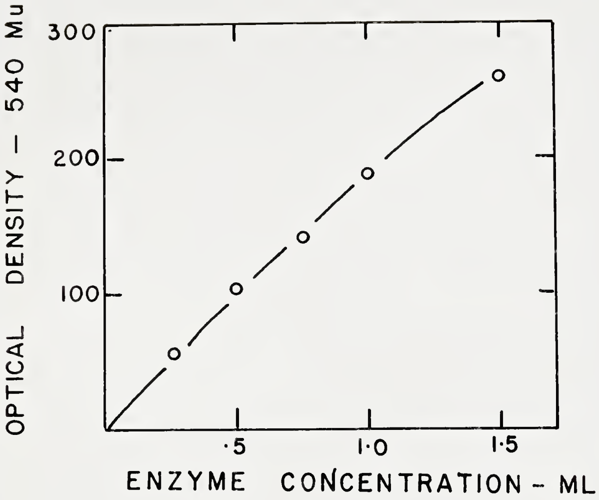


Fig. 15. Inversion of sucrose as a function of concentration of wheat stem invertase. Optical density of 200 equivalent to a production of 25 mg. of invert sugar (as glucose).

(a) pH requirement

No attempt was made to determine the precise pH optimum, since this apparently lies over a very broad range (2). Instead, a test was made to find if the phosphate-citrate buffer of pH 4.7 used in enzyme extraction was suitable for transfructosidase determinations. For this purpose, a series of enzyme reaction mixtures were prepared buffered between pH 4.7 and pH 6.0. Tubes contained 1.0 ml. of

enzyme, 0.5 ml. of phosphate-citrate buffer, and 1.5 ml. of 20% sucrose.

Two or three drops of toluene were added to each tube, followed by incubation at 28° C. for 8 hours. The tubes were then removed and the enzyme inactivated by a two minute treatment in a boiling water bath. Filtrate from the tubes was then spotted on a 20 cm. circular chromatogram by the method of Giri (49) and the chromatogram developed with butanol acetic-water solvent for 5 hours. Results from the chromatogram are shown in Fig. 16.

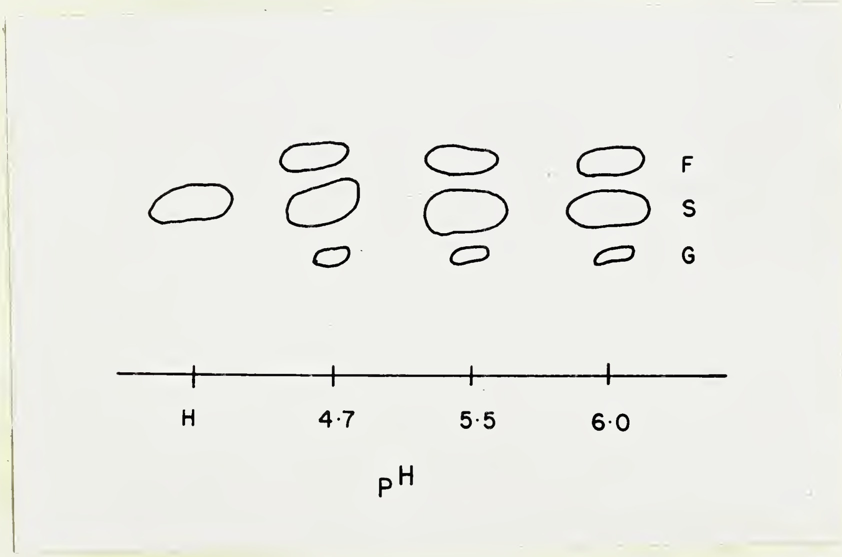


Fig. 16. The affect of pH upon transfructosidase action of wheat stem extract. The chromatogram was sprayed with naphthoresorcinol, hence glucose does not appear. H, heated enzyme; F, S, G, fructose, sucrose, and glucofructan. Stem extract from plants cut on June 5.

Fig. 16 shows that the amount of glucofructan synthesized at pH 4.7 is at least as great as at pH 5.5 or 6.0. All subsequent tests were, therefore, carried out at pH 4.7.

(b) Progress of fructose transfer

Again no precise progress curve for transfructosidase activity was determined because of the very slow rate of reaction. Rather, the shortest period required to produce measurable quantities of glucofructan was determined. For this purpose reaction mixtures were again prepared in test tubes and incubated for periods up to 24 hours. Digests contained 1.0 ml. enzyme, 0.5 ml. of pH 4.7 buffer, 1.5 ml. 20% sucrose, and several drops of toluene. At intervals, tubes were removed, the digests inactivated by heat and the filtrates spotted on a 20 cm. circular chromatogram. Following development for 5 hours, the results shown in Fig. 17 were produced.

The figure indicated a maximum synthesis of glucofructan around 11.5 hours, with a slight reduction at 24 hours. In subsequent determinations, a reaction time of 7.5 hours was chosen as a compromise between the period of instantaneous reaction rate and that for practical analysis.

Concentration curve

A series of enzyme digests were prepared in test tubes containing from 0.25 to 1.5 ml. of enzyme, and 1.5 ml. of

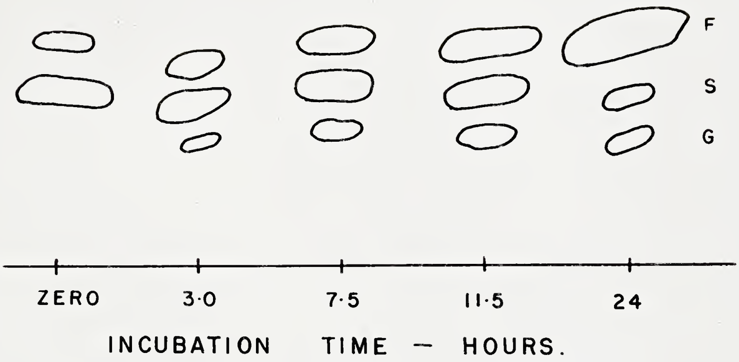


Fig. 17. Progress of transfructosidase synthesis by wheat stem extract. The chromatogram was sprayed with naphthoresorcinol, hence glucose is not shown. Stem extracts from plants cut on June 10.
F, S, G : fructose, sucrose and glucofructan.

20% sucrose. The volume was then made up to 3.0 ml. with pH 4.7 phosphate-citrate buffer, and several drops of toluene added to each tube. Incubation followed for a period of 7.5 hours at 28° C., and then a heat inactivation treatment of 2 minutes at 100° C. Heat inactivated digests were included as controls.

Chromatographic separation and analysis of the synthesized oligosaccharide was then carried out by the method

described previously for examination of hexose ratios of glucofructans. A total of 0.05 ml. filtrate from the enzyme digests was spotted on Whatman No. 3 MM paper. Overnight development was more than adequate for separation. The trisaccharide was eluted in a total volume of 2.4 ml. A corresponding strip was removed from chromatograms of inactive digests, and eluted similarly. Hydrolysis of the trisaccharide was performed by heating 1.0 ml. of eluate with 0.25 ml. of 0.5N H_2SO_4 for a period of 5 minutes in a boiling water bath. The tubes were then cooled and neutralized with 0.5N NaOH. Analysis of reducing sugars was then carried out as usual by Nelson's colorimetric procedure. The enzyme concentration curve produced from the results is shown in Fig. 18.

From the linear nature of the curve shown in Fig. 18, the rate of reaction appears limited only by enzyme concentration. In subsequent analysis, an enzyme concentration of 1.9 ml. was utilized since this falls well within the range of linear response.

Simultaneous Determination of Invertase and Transfructosidase Activity of Leaves and Stems.

Samples of leaves and stems were taken from field grown plants over a period extending from June 5 to August 12. Invertase activity (rate of sucrose hydrolysis) and

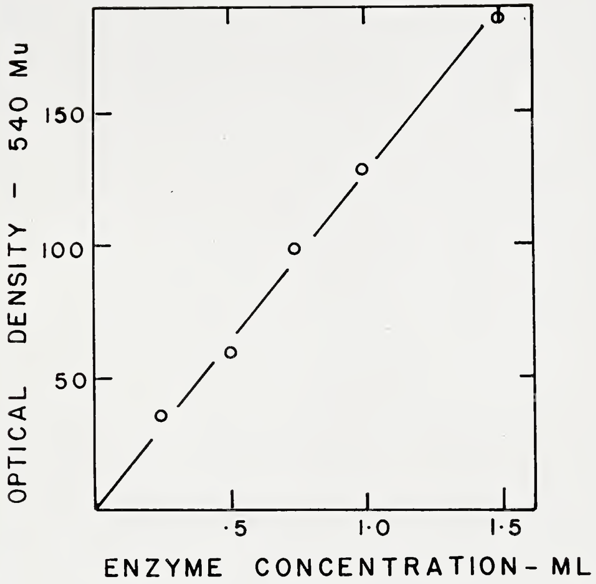


Fig. 18. Transfructosidase synthesis as a function of enzyme concentration.

transfructosidase activity were determined on extracts of leaves and stems by the methods already described. The results of analyses are given in Table 3.

The results of this table indicate a relatively stable invertase/ transfructosidase ratio of wheat leaves, and an extreme divergence of this ratio in wheat stems. This divergence results from a decrease in invertase, and not from an increase in transfructosidase activity. Thus, the data

TABLE III

THE RELATIVE INVERTASE AND TRANSFRUCTOSIDASE ACTIVITY OF LEAF AND STEM EXTRACTS

	Date of Sampling	Per cent Initial Reducing Sugar	Relative Enzyme Activity		
			A-Invertase	B-Transfructo-sidase	Ratio B/A
Leaf	June 9	0.65	60	185	3.8
	18	1.25	110	500	4.6
	25	1.52	93	418	4.5
	August 4	1.05	106	419	4.0
	12 #				
Stem	June 18	2.34	76	335	4.4
	25	4.45	79	386	4.9
	August 4	4.90	18	305	16.8
	12	3.36	3	189	63.0

# Leaves were yellow at this period, hence no extract was available

may be interpreted in two ways: either a specific inhibitor for invertase is present in stems, or two separate enzymes are involved. Possibly invertase inhibitors exist in the high content of reducing sugars initially present in stem extracts.

To test the latter hypothesis, the stem extract showing the widest divergence in invertase transfructosidase activity was dialyzed, and the invertase activity re-determined. Dialysis was carried out in a cellophane bag against distilled water at 5° C. for a period of 4 hours.

Reducing sugar content of the leaf extract was lowered from 3.36% to .08% by this treatment. Invertase activity, on the other hand, rose from a relative value of 3.0 to a value of 20.

Although the above evidence indicates a dializable factor in stems which specifically inhibits invertase, no conclusive proof was obtained that the factor involved was actually that of reducing sugar.

Transfructosidase Activity at High Concentrations of Stem Extract

In an attempt to demonstrate the synthesis of higher polymer glucofructans, transfructosidase activity was examined with the stem extract diluted as little as possible. Additions of fructose and glucose were made, since Edelman (41)

found that presence of these sugars favoured transfructosidase activity over sucrose hydrolysis. Sugar solutions of 40% were prepared by dissolving the sugars in phosphate-citrate buffer of pH 4.7. Stem extract was prepared by grinding and pressing the stems without addition of buffer. The final concentration of sugars in digests containing 1 ml. of extract were as follows:

(a) 10% sucrose
(b) 10% sucrose plus 5% fructose
(c) 10% sucrose plus 5% fructose plus 5% glucose

Digests were incubated at room temperature, and the progress followed over a 24 hour period by paper chromatography.

The chromatograms produced from these experiments showed no indication of synthesis of glucofructans other than the usual trisaccharide.

Transfructosidase Activity in Presence of ATP and Phosphorylated Sugars

As mentioned previously, a phosphorylative mechanism for glucofructan synthesis seems unlikely, since the intermediate required, fructose-2 phosphate, has not been identified in plants. Nevertheless, the compound may be an unstable form recurrently synthesized in the plant. The activity of adenosine triphosphate, or transphosphorylation

offers a possible synthetic mechanism. To test these possibilities, transfructosidase activity was determined in stem extracts containing ATP and phosphorylated sugars.

Adenosine triphosphate experiments were performed under conditions similar to those described by Ling, Byrne and Lardy (72) for phosphohexokinase. Experiments involving phosphorylated sugars were performed under conditions similar to those outlined by Morton (82) for transphosphorylation by phosphatases.

The digests were prepared as follows:

(a) 1.0 ml. stem extract (prepared in citrate buffer pH 5.0)
    1.5 ml. 20% sucrose, 5% fructose
    0.2 ml. sodium arsenate
    0.3 ml. magnesium nitrate
    0.2 ml. of .02 M ATP

(b) 1.0 ml. stem extract (prepared in citrate buffer pH 5.0)
    1.5 ml. 20% sucrose, 5% fructose
    0.3 ml. magnesium nitrate
    0.1 M fructose-1 phosphate, fructose-1-6 diphosphate, or glucose-1 phosphate.

Digests were incubated at room temperature after addition of a few drops of toluene and the progress of transfructosidase activity followed chromatographically over a 24 hour period.

No indication of synthesis of higher polymer

glucofructans was shown by the chromatograms produced from the digest containing phosphorylated sugars on adenosine triphosphate.

Accumulation of Glucofructans in Wheat Stems

The accumulation of glucofructans in wheat stems was examined at stages of growth from heading to maturity. In addition to Golden Ball, two varieties of *T. vulgare* Vill. were studied: Thatcher and Rescue. Stem samples were taken from field grown plots at 10 a.m. on sunny days. The sugars were extracted and purified as already described. Separations were performed in the usual manner on Whatman No. 4 paper. Alcohol soluble glucofructans were eluted from the paper en masse, and determined quantitatively by Nelson's procedure (84). Alcohol insoluble glucofructan was treated similarily. Total sugar was determined as the sum of glucofructan fractions, plus the fructose, glucose and sucrose eluted from the same chromatogram. The results of analysis for alcohol soluble glucofructans are shown in Fig. 19; for water soluble glucofructan and total sugars, in Fig. 20. The percentage total sugars of wheat stems in the form of glucofructan at maximum concentration is shown in Table 4.

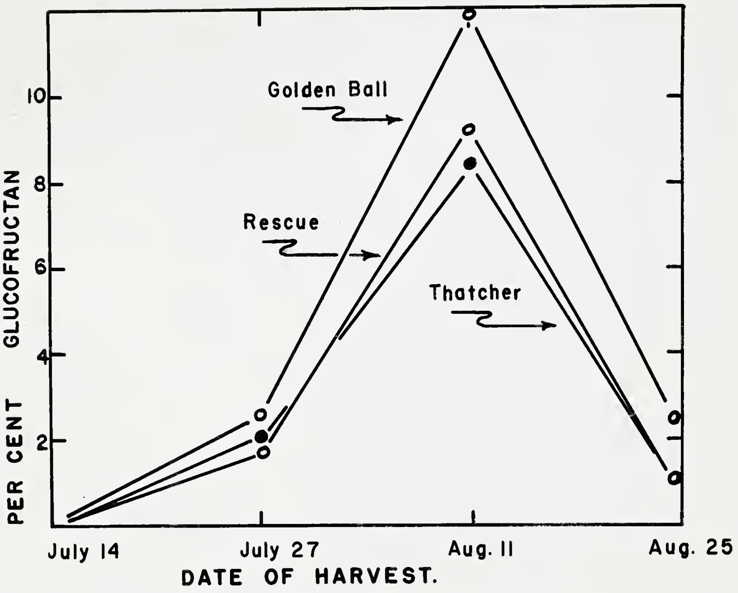


Fig. 19. Accumulation of alcohol soluble glucofructans in wheat stems.

TABLE IV

PER CENT GLUCOFRUCTANS AND PER CENT TOTAL SUGARS IN WHEAT STEMS AT MAXIMUM SUGAR CONCENTRATION (AUGUST 11)

Wheat Variety	Glucofructan Alcohol Soluble	Glucofructan Water Soluble	Total Glucofructans	Total Sugars
Golden Ball	12.0	3.0	15.0	24.0
Rescue	9.0	1.7	10.7	13.0
Thatcher	8.5	1.7	10.2	19.0

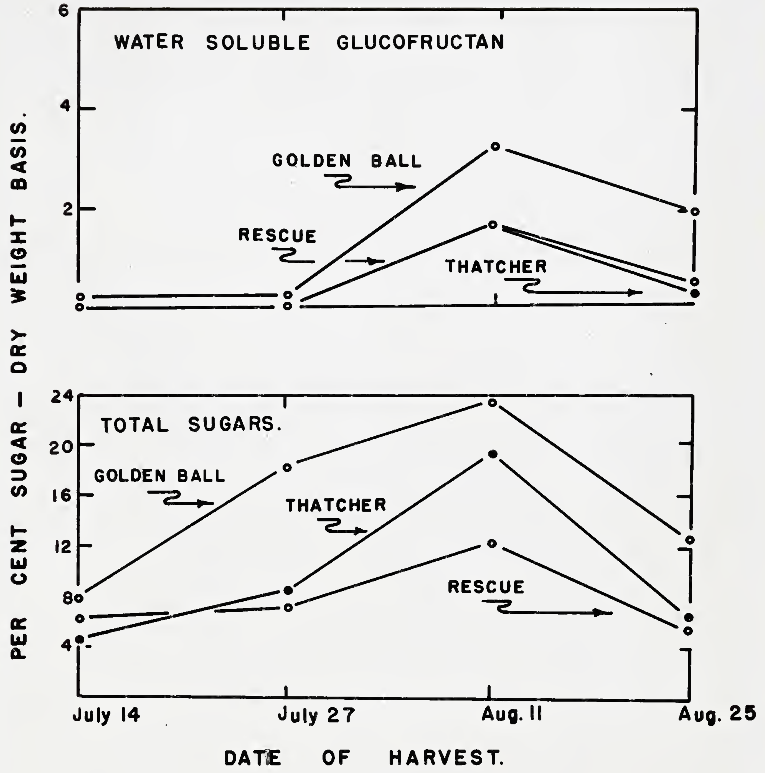


Fig. 20. Accumulation of water soluble glucofructan and total sugars in wheat stems.

The period of July 14, shown in Fig. 19, corresponds roughly to the time of heading, while August 11 corresponds to the milk stage. Fig. 19 illustrates that accumulation of alcohol soluble glucofructans commences about the time of heading, reaches a peak at the milk stage, and then declines rapidly as the plant matures. Fig. 20 shows much the same pattern for water soluble glucofructan and for total sugars.

The results from Table IV indicate that at the period of maximum concentration more than half the total sugar of wheat stems is in the form of glucofructan.

DISCUSSION

Unequivocal proof of structure of the oligosaccharides of wheat stems must await crystallization of the compounds in sufficient quantity to allow determination by classical methods. Nevertheless, sufficient information has been obtained to identify, tentatively, two stem trisaccharides as F^1-B-fructosylsucrose and F^6-B-fructosylsucrose. It seems reasonably certain also that the higher polymer, alcohol soluble, glucofructans are either members of two homologous series based upon these trisaccharides, or, alternatively, closely related compounds.

Although no direct evidence was obtained that a sucrose unit is present in the water soluble glucofructan chain, features of invertase and acid hydrolysis suggest this possibility. It cannot be said for certain that this substance is a fructose polymer of a lower glucofructan.

Judging from the extremely high sugar content of stems toward maturity, wheat stems function not only in support and translocation, but also as storage organs for sugars. It seems likely that accumulation of glucofructans is facilitated by an active synthetic mechanism, and by inhibition of the enzyme invertase which normally brings about glucofructan hydrolysis. This view is supported by the evidence indicating that an inhibitor of invertase is

present in maturing wheat stems.

The nature of this inhibitor remains unknown. Fructose and glucose are known to inhibit sucrose hydrolysis by invertase, without affecting fructose transfer (41). The magnitude of inhibition is low compared to that which occurs in stems at roughly comparable sugar concentration. Recently Srinivasan and Quastel (104) reported sucrose hydrolysis by invertase is competitively inhibited by glucose-amine. Since this compound functions as a fructose acceptor, the oligosaccharides formed under these conditions contained glucose-amine.

Failure to demonstrate synthesis of more than one oligosaccharide by transfructosidase activity suggests that a second enzyme system is required for synthesis of higher glucofructans. It seems unlikely that such a synthesis occurs through polymerization of fructose phosphate, judging from the negative results reported here.

An alternative synthesis for higher polymers perhaps exists in a mechanism similar to the uridine diphosphate glucose synthesis of sucrose. Neish (83) has recently reviewed the evidence supporting such an enzyme mechanism for synthesis of xylan and cellulose in the wheat plant. A drawback to glucofructan synthesis by this mechanism is the apparent requirement of fructose-2 phosphate for formation of uridine diphosphate fructose.

BIBLIOGRAPHY

1. Adams, M., N.K. Richtmyer and H.C. Hudson. Some enzymes present in highly purified inverttase preparations: a contribution to the study of fructofuranosidases, galactosidases, glucosidases and mannosidases. J. Am. Chem. Soc. 65, 1369-1383 (1953).

2. Allen, P.J., and J.S.D. Bacon. Oligosaccharides formed from sucrose by fructose-transferring enzymes of higher plants. Biochem. J., 63, 200-206, 1956.

3. Alm, R.S. Gradient elution analysis 2, Oligosaccharides. Acta. Chem. Scand. 6, 1186-1193, (1952).

4. Alm, R.S., R.J.P. Williams and A. Tiselius. Gradient elution analysis. Acta. Chem. Scandia. 6, 826-836 (1952).

5. Archbold, H.K. Saccharase activity in the barley plant. Biochem. J. 34, 749-763 (1940).

6. Ash, A.S.F., and T.M. Reynolds. Ketose oligosaccharides in the apricot fruit. Nature 174, 602-603 (1954).

7. Aspinall, G.O., and R.G.J. Telfer. Studies on fructosans. Part IV. A fructosan from Dactylis glomerata. J. Chem. Ind. 1822 (1951).

8. Babbitt, J.M. Periodate oxidation of carbohydrates. Advances in Carbo. Chem. 11, 1-40 (1956).

9. Bacon, J.S.D. Transfructosidation by a yeast invertase preparation. Biochem. J. 50, XVIII (1952).

10. Bacon, J.S.D. Ann. Rep. Chem. Soc. 50, 281 (1953) Cited by J.S.D. Bacon, Biochem. J. 63, 200-206, (1956).

11. Bacon, J.S.D. The oligosaccharides produced by the action of yeast invertase preparations on sucrose. Biochem. J. 57, 320-328 (1954).

12. Bacon, J.S.D. Methods for measuring transglycosydase activity of invertase. Methods in Enzymology 1, 258-262. Ed. Colowick, S.P. and N.O. Kaplan. Academic Press, N.Y. 1955.

13. Bacon, J.S.D. and J.D. Bell. A new trisaccharide produced from sucrose by mould invertase. J. Chem. Soc. 2528-2530 (1953).

14. Bacon, J.S.D., and J. Edelman. Arch. Biochem. 28, 467 (1950). Cited by J.S.D. Bacon, Biochem. J. 57, 320-328 (1954).

15. Bacon, J.S.D., and J. Edelman. The carbohydrates of the Jerusalem artichoke. Biochem. J., 48, 114-128 (1951).

16. Balston, J.N., and B.E. Talbot. A guide to filter paper and cellulose powder chromatography. H. Reeve Angel and Co. 1952.

17. Bathurst, N.O., and R.M. Allison. The preparation of plant tissue for analysis. New Zealand J. Sc. Tech. B, 31, No.2, 2-14 (1949).

18. Bayly, R.J. and E.J. Bourne. A new method for the paper chromatography of oligosaccharides. Nature 171, 385-387 (1953).

19. Bealing, F.J. Mould "glucosaccharase", a fructosidase. Biochem. J. 55, 93-101 (1953).

20. Bealing, F.J., and J.S.D. Bacon. The action of mould enzymes on sucrose. Biochem. J. 53, 277-285 (1953).

21. Bell, D.J., and J. Edelman. Disaccharide synthesis following fructose-transfer from sucrose by yeast invertase. J. Chem. Soc. 4652-4654 (1954).

22. Bhatia, T.S., M.N. Satyanarayana, and M. Srinivasan. Carbohydrates of Agave vera Cruz. Curr. Sci. 22, 16-17 (1953).

23. Bhatia, I.S., M.N. Satyanarayana and M. Srinivasan. Transfructosidase from Agave vera cruz Mill. Biochem. J. 61, 171-174 (1955).

24. Bidwell, R.G.S., G. Krotkov, and G.B. Reid. Paper chromatography of sugars in plants. Can. J. Bot., 30, 291-305 (1952).

25. Bishop, C.T. Oligosaccharides of Xylose from wheat straw hemicellulose. Can. J. Chem., 33, 1073-1078 (1955).

26. Blanchard, P.H., and N. Albon. Arch. Biochem. 29, 220 (1950). Cited by J.S.D. Bacon, Biochem. J. 57, 320-328 (1954).

27. Blish, M.J., R.M. Sandstedt, and G.R. Astleford. Sugars, diastatic activity, and "gassing power" in flour. Cereal Chem. 9, 378-393 (1932).

28. Boggs, L., L.S. Cuendet, T. Ehrenthal, R. Koch, and F. Smith. Separation and identification of sugars using paper chromatography. Nature 166, 520-521 (1950).

29. Boggs, L.A., and F. Smith. The constitution of the glucofructan of the tuber of the Hawaiian "Ti" plant. J. Amer. Chem. Soc. 78, 1880-1885 (1956).

30. Bradfield, A.E., and Flood, A.E. Soluble carbohydrates of fruit plants. Nature 166, 264-265 (1950).

31. Brown, F. Periodate and group assay of polysaccharides. Nature 156, 785 (1945).

32. Brown, F., E.L. Hirst, L. Hough, J.K.N. Jones and H. Wadman. Separation and identification of methylated sugars on the paper chromatogram. Nature 161, 720 (1948).

33. Brown, F., Sonia Dunstan, T.G. Halsall, E.L. Hirst, and J.K.N. Jones. Applications of new methods of end-group determination to structural problems in the polysaccharides. Nature 156, 785-786 (1945).

34. Browne, C.A. and Zerban, F.W. Physical and chemical methods of sugar analysis. 3rd ed. John Wiley & Sons, New York. 1948.

35. Buchanan, J.G., C.A. Dekker and A.G. Long. Detection of non-reducing carbohydrates. Brit. Med. Bull. 10, No.3, (1954).

36. Burton, K. and H.A. Krebs. The free energy changes associated with the individual steps of the tricarboxilic acid cycle, glycolysis and alcoholic fermentation and with the hydrolysis of the pyrophosphate groups of adenosinetriphosphate. Biochem. J. 54, 94-107 (1953).

37. Dehn, U.M., K.E. Jackson, and D.A. Ballard. Identification of common carbohydrates. Ind. Eng. Chem. Anal. Ed. 4, 413-415 (1932).

38. Dimler, R.J., W.C. Shaefer, C.S. Wise and C.E. Rist. Quantitative paper chromatography of D-glucose and its oligosaccharides. Anal. Chem. 24, 1411-1414 (1952).

39. Doudoroff, M. and R. O'Neal. On the reversibility of levulan synthesis by Bacellus subtilis. J. B.C. 159, 585-592 (1945).

40. Edelman, J. Fructose polysaccharides in plants, PhD. Thesis, University of Sheffield, 1950.

41. Edelman, J. Transfer reactions catalysed by some sucrose preparations. Biochem. J. 57, 22-33 (1954).

42. Esau, Katherine. Plant Anatomy. John Wiley & Sons, New York. 1953.

43. Euler, H. and K. Myrbach. Kinetesche Untersuchungen an Saccharase. Z. physiol. Chem. 124, 159-170 (1923).

44. Feingold, D.S., A. Avigad and S. Hestrin. The mechanism of polysaccharide production from sucrose. 4. Isolation and probable structures of oligosaccharides formed from sucrose by a levansucrose system. Biochem. J. 64, 351-361 (1956).

45. Fisher, E.H., L. Kohtes, and J. Fellig. Helv. Chem. Acta. 34, 1132- (1951).

46. Flood, A.E., E.L. Heist and J.K.N. Jones. Quantitative analysis of mixtures of sugars by the method of partition chromatography. Part I. Standardization of Procedure. J. Chem. Soc., 1678 (1948).

47. French, D. Structure of Pan's crystalline trisaccharide. Science 113, 352-353 (1951).

48. French D. and G.M. Wild. Correlation of carbohydrate structure with paper-gram mobility. J. Am. Chem. Soc. 75, 2612-2616 (1953).

49. Giri, K.V. Preparative circular paper chromatography. Nature 173, 1194-1195 (1954).

50. Gross, D. Paper electrophoresis of sugars at high potential gradients. Nature 172, 908-909 (1953).

51. Gross, D. Paper electrophoresis of the oligosaccharides synthesized from sucrose by yeast invertase. Nature 173, 487-489 (1954).

52. Gross, D., P.H. Blanchard and D.J. Bell. Neokestose: a trisaccharide formed from sucrose by yeast invertase. J. Chem. Soc. 1727-1730 (1954).

53. Halsall, T.G., E.L. Hirst and J.K.N. Jones. Oxidation of carbohydrates by the periodate ion. J. Chem. Soc. 1427-1432 (1947).

54. Hanes, C.S. Proc. Roy. Soc. (London) B. 128, 421; 129, 174 (1940). Cited by R.L. Whistler and C.S. Smart. Polysaccharide Chemistry. Academic Press, New York. 1953.

55. Hehre, E.J. Production from sucrose of a serologically reactive polysaccharide by a sterile bacterial extract. Science 93, 237-238 (1941).

56. Hehre, E.J. Enzymic Synthesis of Polysaccharides: a biological type of polymerization. Adv. Enzymol. 11, 297-319 (1951).

57. Hestrin, S., and S. Avineri-Shapiro. The mechanism of polysaccharide production from sucrose. Biochem. J. 38, 2-10 (1944).

58. Hestrin, S., D.S. Feingold and G. Avigad. The mechanism of polysaccharide production from sucrose 3. Donor-acceptor specificity of levansucrase from Aerobacter Levanicum. Biochem. J. 64, 340-351 (1956).

59. Hirst, E.L. Some aspects of the chemistry of fructosans. Proceedings of the Chem. Soc. (July) 193-204 (1957).

60. Hirst, E.L. and J.K.N. Jones. The application of partition chromatography to the separation of the sugars and their derivatives. Discussions of the Faraday Soc. No. 7, 268-278 (1948).

61. Horrocks, R.H. Paper partition chromatography of reducing sugars with benzidine as a spraying agent. Nature 164, 444 (1949).

62. Hough L., J.K.N. Jones and U.H. Uadman. Quantitative analysis of mixtures of sugars by the method of partition chromatography. Part IV. The separation of the sugars and their methylatea derivatives on columns of powdered cellulose. J. Chem. Soc. 2511-2516 (1949).

63. Janssen, G. Physical measurements of the wheat plant at various stages of its development. Plant Physiol. 4, 477-491 (1929).

64. Jones, J.K.N. and F. Smith. Plant gums and mucilages. Adv. Carbo. Chem. 4, 243-291 (1949).

65. Kalckar, H.M. The Mechanism of Enzyme Action, p.699. Ed. by McElroy, U., and B. Glass. Baltimore: The John Hopkins Press. 1954.

66. Koch, R.B., U.F. Geddes, and F. Smith. The carbohydrates of Gramineae. I. The sugars of the flour of wheat (Triticum vulgare). Cereal Chem. 28, 424-430 (1951).

67. Kohanyi, G. and R. Dedonder. Oligosaccharides formed by an enzyme of Bacillus subtilis. C.R. Acad. Sci., Paris, 233, 1142 (1951).

68. Krotkov, G. Diurnal changes in the carbohydrates of wheat leaves. Can. J. Res. Sec. C, 21, 26-34 (1943).

69. Laidlaw, R.A. and S.G. Reid. Filter-paper chromatography: Extraction of sugars from the paper at room temperature. Nature, 166, 476-477 (1950).

70. Laidlaw, R.A. and S.G. Reid. Analytical studies on the carbohydrates of grasses and clovers. I. Development of methods for the estimation of the free sugar and fructosan contents. J. Sci. Food Ag., 1, 19-25 (1952).

71. Laidlaw, R.A. and S.G. Reid. Analytical studies on the carbohydrates of grasses and clovers. III. Carbohydrate breakdown during wilting and storage. J. Sci. Food Agric. 4, 527-531 (1953).

72. Ling, K.H., W.L. Byrne, and H. Lardy. Phosphohexokinase. Methods in Enzymology. 1, 306-310. Ed. S.P. Colowick and N.O. Kaplan, Academic Press, New York. 1955.

73. Levi, I. and C.B. Purves. The structure and configuration of sucrose. Advanc. Carbo. Chem. 4, 1-35 (1949).

74. Lindberg, B. and B. Wickberg. Separation of methylated sugars on carbon columns. Acta. Chem. Scandia 8, 569-573 (1954).

75. Lopatecki, L.E., E.L. Longair, and C.W. Forstad. Soluble carbohydrates of wheat stems and leaves. Can. J. Bot., 35, 9-12 (1957).

76. MacLeod, Anna M. Studies on the free sugars of the barley grain. IV. Low molecular weight fructosans. J. Inst. Brew., LIX, 462-469 (1953).

77. McFarren, E.F., K. Brand and H.R. Rutkowski. Quantitative determination of sugars on filter paper chromatograms by direct photometry. Anal. Chem. 23, 1146-1149 (1951).

78. Manners, D.J. The enzymatic degradation of polysaccharides. Quart. Reviews 9, 73 (1955).

79. Michaelis, L. and Menton, M.L. Die kinetic der invertinwirkung. Biochem. Z., 49, 333-369 (1913).

80. Mizuno and Kaneo Hayashi. A new crystalline glucofructan. J. Agr. Chem. Soc. Japan, 28, 78-83 (1954). Cited Chem. Ab. 4802 (1955).

81. Moore, S. and P. K. Link. Carbohydrate characterization. I. The oxidation of aldoses by hypoiodite in methanol. J. Biol. Chem. 133, 293-299 (1940).

82. Morton, R.K. Transphosphorylation by phosphatases. Methods in Enzymology, 2, 556-561. Ed. S. P. Colowick and N. O. Kaplan. Academic Press, New York, 1955.

83. Neish, A. C. The biosynthesis of cell wall carbohydrates. IV. Further studies on cellulose and xylan in wheat. Can. J. Biochem. and Physiol. 36, p. 187-193. 1958.

84. Nelson, N. Photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 153, 375-380 (1944).

85. Neuberg, C. and Strauss, E. Quantitative formation of osazones. Arch. Biochem. 11, 457-465 (1946).

86. Newton, R. and W.R. Brown. Seasonal changes in the composition of winter wheat plants, in relation to frost resistance. Jour. Agric. Sci. 16, 522-538 (1926).

87. Partridge, S.M. Application of the paper partition chromatogram to the quantitative analysis of reducing sugars. Nature 158, 270-271 (1946).

88. Partridge, S.M., with a note by R. G. Westall. Filter-paper partition chromatography of sugars. Biochem. J. 42, 238-250 (1948).

89. Partridge, S.M. Filter-paper partition chromatography of sugars. Biochem. J. 42, 251-253 (1948).

90. Percival, J. The wheat plant. E.P. Dutton and Co. New York, 1921.

91. Perlin, A.S. Oxidation of carbohydrates with periodate in the Warburg respirometer. J. Am. Chem. Soc. 76, 4101-4103 (1954).

92. Perlin, A.S. Structure of reducing disaccharides by lead tetraacetate oxidation. Anal. Chem. 27, 396-399 (1955).

93. Phillips, J.D. and A. Pollard. Degradation of sugars on ion-exchange columns of Amberlite 1 RA400(OH). Nature 171, 41-42 (1953).

94. Pigman, W.W., and R.M. Goepp. Chemistry of the carbohydrates. Academic Press Inc. New York, 1948.

95. Platt, A.W. The influence of some environmental factors on the expression of the solid stem character in certain wheat varieties. Sci. Agric. 21, 139-151 (1941).

96. Platt, A.W. and Ruby Larson. An attempt to transfer solid stem from Triticum durum to T. vulgare by hybridization. Sci. Agric. 24, 214-220 (1944).

97. Potter, A. and W.Z. Hassia. Starch I. End-group determination of amylose and amylopectin by periodate oxidation. J. Amer. Chem. Soc. 70, 3488-3490 (1948).

98. Raybin, H.W. A new colour reaction with sucrose. J. Amer. Chem. Soc. 55, 2603-2604 (1933).

99. Raybin, H.W. The direct demonstration of the sucrose linkage in the oligosaccharides. J. Amer. Chem. Soc. 59, 1402-1403 (1937).

100. Roe, J.H. A colourimetric method for the determination of fructose in blood and urine. J. Biol. Chem. 107, 15-22 (1934).

101. Robbins, W.W. The botany of crop plants. P. Blakiston's Son & Co. Philadelphia, 1917.

102. Roberts, D.W.A. Qualitative and quantitative determination of wheat leaf carbohydrates. Can. J. Res., C. 28, 754-779 (1950).

103. Roberts, D.W.A. Physiological and biochemical studies in plant metabolism. VIII. Distribution of invertase in the first leaf of Khapli wheat. Can. J. Bot. 31, 367-382 (1953).

104. Srinivasan, S. and J.H. Quastel. Enzymic synthesis of oligo- and polysaccharides containing D-glucosamine. Science 127, 143-144 (1958).

105. Van Slyke, D.D. The kinetics of hydrolytic enzymes and their bearing on methods for measuring enzyme activity. Adv. in Enzymology, 2, 33-47 (1942).

106. Wagner, H.G. An improved copper reduction method for the micro-determination of reducing sugars. Analyst 79, 34-38 (1954).

107. Ward, G.M. Physiological and biochemical studies in plant metabolism. VII. The invertase of the seedling wheat leaf. Can. J. Bot. 31, 81-89 (1953).

108. Ward, G.M. The isolation and identification of sucrose from the seedling wheat leaf. Can. J. Bot. 31, 75-80 (1953).

109. de Whalley, H.C.S. and D. Gross. Determinations of raffinose and kestose in plant products. Methods of Biochem. Analysis 1, 307-339, Interscience Pub., New York. 1951.

110. Whelan, W.J. and D.M. Jones. B-methyl fructoside as a substrate in transfructosylation. Biochem. J. 54, XXXIV (1953).

111. Whistler, R.L. and D.F. Durso. Chromatographic separation of sugars on charcoal. J. Amer. Chem. Soc. 72, 677-679 (1950).

112. Whistler, R.L. and D.I. McGilvray. Chemistry of the carbohydrates. Ann. Rev. Biochem. 23, 79-98 (1954).

113. Whistler, R.L. and C.L. Smart. Polysaccharide Chemistry. Academic Press Inc. 1953.

114. White, L.M. and G.E. Secor. Occurrence of two similar homologous series of oligosaccharides in wheat flour and wheat. Arch. Biochem. Biophics, 43, 60-66 (1953).

115. Williams, K.T. and A. Bevenue. Use of ion-exchange resins for removal of non-sugar reducing substances in the analysis of fresh leafy plant materials for sugars. J. Ass. Offic. Agric. Chem. Wash., 34, 817-821 (1951).

116. Williams, K.T. and A. Bevenue. The chromatographic examination of sugars in wheat flour. Cereal Chem. 28, 416-423 (1951).

117. Williams, K.T. and A. Bevenus. Use of ion exchange resins for removal of non-sugar reducing substances in the analysis of fresh leafy plant material for sugars. J. Ass. Off. Agric. Chem. Wash., 34, 817-821 (1951).

118. Williams, K.T. and E.F. Potter. Sugars in plants. J. Assoc. Offic. Agr. Chemists, 36, 401-404 (1953).

119. Wylam, C.B. Analytical studies on the carbohydrates of grasses and clovers. IV. Further developments in the methods of estimation of mono, di, and oligosaccharides and fructosan. J. of Sc. Food Agric. 5, 167-172 (1954).

120. Zimmermann, M.H. Translocation of organic substances in Trees. I. The nature of the sugars in the sieve tube exudate of trees. Plant Phys. 32, 288-291 (1957).

121. Zimmermann, M.H. Translocation of organic substances in trees. II. On the translocation mechanism in the phloem of white ash (Fraxinus americana L.). Plant Physiol. 32, 399-404 (1957).

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